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(54) Title: SURFACE COMPLEXED LYMPHOTOXIN

(57) Abstract

This invention relates to a membrane associated protein, p33, and the complex it forms with a soluble lymphotoxin. These proteins are found on the surface of a number of cells, including phorbol ester (PMA) stimulated T cell hybridoma II-23.D7 cells. These proteins and complexes are useful as anti-inflammatory agents, enhancers of tumor infiltrating lymphocytes, tumor growth inhibiting agents, T cell inhibiting agents and T cell activating agents.

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SURFACE COMPLEXED LYMPHOTOXIN

TECHNICAL FIELD OF THE INVENTION

This invention relates to a protein produced by lymphocytes and other cell types and the complex it forms with lymphotoxin (LT). More particularly, it relates to a membrane-associated protein, p33, identified on the surface of T lymphocytes, T cell lines, and lymphokine-activated killer cells, and to the complex formed when p33 binds to LT. The p33 protein of this invention is expected to be useful in holding LT formed within the cell on the cell surface where the p33/LT complex may act as an inflammation regulating agent, a tumor growth inhibiting agent, a T cell inhibiting agent, a T cell activating agent or an HIV regulating agent. Furthermore, the antitumor activity of a p33/LT complex may be delivered to tumor cells by tumor infiltrating lymphocytes (TILs) transfected with the gene for p33.

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BACKGROUND OF THE INVENTION

Tumor necrosis factor (TNF) and lymphotoxin (LT) are soluble proteins noted originally for their ability to inhibit the growth of tumors [L. Old, "Tumor Necrosis Factor", Science, 230, 630 (1985)]. Further research demonstrated that both proteins exhibit a wide range of activities. TNF, for example, appears to play a major role in specific aspects of metabolic control, in the response to endotoxin shock, and in the regulation of hematopoietic cell development. [B. Beutler et al., "The History, Properties, and Biological Effects of Cachectin", Biochemistry, 27, (1988); M. Akashi et al., "Lymphotoxin: Stimulation And Regulation of Colony Stimulating Factors in Fibroblasts", Blood, 74, 2383 (1989); G. Roodman et al., "Tumor Necrosis Factor-alpha and Hematopoietic Progenitors: Effects Of Tumor Necrosis Factor On The Growth Of Erythroid Progenitors CFU-E And BFU-E And The Hematopoietic Cell Lines k562, HL60, And HEL Cells", Exp. Hematol., 15, 928 (1987)]. Along with IL-1 and IL-6, TNF is also a major mediator of the inflammatory response. [D. Cavender et al., "Endothelial Cell Activation Induced By Tumor Necrosis Factor And Lymphotoxin", Amer. Jour. Path., 134, 551 (1989); R. Cotran et al., "Endothelial Activation Its Role In Inflammatory And Immune Reactions", in Endothelial Cell Biology, (Plenum Press, Simonescu & Simonescu, eds., 1988) 335]. TNF also appears to be involved in T cell activation under certain conditions. [M. Shalaby et al., "The Involvement Of Human Tumor Necrosis Factors-α And-β In The Mixed Lymphocyte Reaction", J. Immun., 141, 499 (1988); N. Damle et al., "Distinct Regulatory Effects of IL-4 and TNF-α During CD3-Dependent and CD3-Independent Initiation Of Human

T-Cell Activation", Lymph. Res., 8, 85 (1989); G. Ranges et al., "Tumor Necrosis Factor-α As A Proliferative Signal For An IL-2-Dependent T Cell Line: Strict Species Specificity of Action", Amer. Assoc. Immun., 142, 1203 (1989); G. Ranges et al., "Tumor Necrosis Factor α/Cachectin Is A Growth Factor For Thymocytes", J. Exp. Med., 167, 1472 (1988); P. Scheurich et al., "Immunoregulatory Activity Of Recombinant Human Tumor Necrosis Factor (TNF)-α: Induction Of TNF Receptors On Human T Cells And TNF-α-Mediated Enhancement Of T Cell Responses", J. Immun., 138, 1786 (1987)].

LT also has many activities, generally similar, but not identical to those of TNF, including tumor necrosis, induction of an antiviral state, activation of polymorphonuclear leukocytes, induction of class I major histocompatibility complex antigens on endothelial cells, induction of adhesion molecules on endothelium and growth hormone stimulation. [N. Ruddle and R. Homer, "The Role of Lymphotoxin in Inflammation", Prog. Allergy, 40, pp. 162-182 (1988)].

In recent years genes for both TNF and LT have been isolated and cloned, leading to their complete characterization and to the availability of recombinant forms of both proteins. [P. Gray et al., "Cloning and Expression of cDNA For Human Lymphotoxin, A Lymphokine With Tumor Necrosis Activity", Nature, 312, pp. 121-124 (1984); D. Pennica et al., "Human Tumor Necrosis Factor: Precusor Structure, Expression And Homology To Lymphotoxin", Nature, 312, 724 (1984)].

TNF is produced by several types of cells, including monocytes, fibroblasts, T cells and Natural Killer (NK) cells. [D. Goeddel et al., "Tumor Necrosis Factors: Gene Structure And Biological Activities", Cold Spring Harbor Symposium Quant. Biol., 51, 597

(1986); D. Spriggs et al., "Tumor Necrosis Factor Expression In Human Epithelial Tumor Cell Lines", J. Clin. Invest., 81, 455 (1988); M. Turner et al., "Human T cells From Autoimmune and Normal Individuals Can Produce Tumor Necrosis Factor", Eur. J. Immun., 17, 1807 (1987)]. Lymphotoxin secretion appears to be a specific property of only activated T cells and certain B-lymphoblastoid tumors. [N. Paul et al., "Lymphotoxin", Ann. Rev. Immun., 6, 407 (1988)].

Investigators have also detected murine and human forms of TNF that are associated with the surface of various cells either as a transmembrane protein or a receptor-bound molecule [B. Luettig et al., "Evidence For the Existence of Two Forms of Membrane Tumor Necrosis Factor: An Integral Protein and a Molecule Attached To Its Receptor", J. Immun., 143, 4034 (1989); M. Kriegler et al., "A Novel Form of TNF/Cachectin Is a Cell Surface Cytotoxic Transmembrane Protein: Ramifications For the Complex Physiology of TNF", Cell, 53, pp. 45-53 (1988); and M. Kinkhabwala et al., "A Novel Addition To the T Cell Repertory", J. Exp. Med., 171, pp. 941-946 (1990)]. Some researchers have also indicated that a membrane-associated form of lymphotoxin may be expressed on the surface of lymphocytes under certain circumstances [J. Hiserodt, et al., "Identification of Membrane-Associated Lymphotoxin (LT) On Mitogen-Activated Human Lymphocytes Using Heterologous Anti-LT Antisera In Vitro", Cell. Immun., 34, pp. 326-339 (1977); C. Ware et al., "Mechanisms of Lymphocyte-Mediated Cytotoxicity", J. Immun., 126, pp. 1927-1933 (1981); U. Anderson et al. J. Immunol. Methods, 123, 233 (1989); Y. Abe et al., Jpn. J. Canc. Res., 82, 23 (1991)].

We have now identified a novel surface protein, p33, that targets LT produced in the cell to

the cell membrane where p33 and LT appear as a complex (designated "p33/LT" throughout this disclosure). The p33/LT complex may exhibit similar cytolytic and cell regulatory activity to the soluble LT and TNF proteins. The p33 protein may represent a novel receptor specific for LT (p33 does not bind TNF). The membrane-associated p33 complexed with LT may represent, as a complex, a novel ligand for T cell interactions with other cells and may also be useful in targeted cell lysis.

SUMMARY OF THE INVENTION

The novel protein of the present invention has been named p33. This protein is found on the surface of several types of lymphocyte cells, including OKT3-stimulated primary T cells, antigen-specific IL-2 dependent CTL clones, and a PMA-stimulated human T cell hybridoma, II-23.D7. It forms a novel complex with LT.

p33 has a molecular weight of 31-35 kD as determined by immunoprecipitation and SDS-PAGE and exhibits N-linked glycosylation. In addition, the protein contains both methionine and cysteine residues, and CNBr cleavage indicates that the methionine residue(s) are located within 1-2 kD of the N-terminus or the C-terminus.

p33 on the cell membrane binds with LT produced in the cell, thus "targeting" the LT to the cell membrane. In the absence of p33, LT is secreted into the pericellular medium. The p33/LT complex and the other polypeptide complexes of this invention (both non-cell associated and on the surface of cells) are recognized by polyclonal antisera, raised against recombinant lymphotoxin (rLT) expressed in CHO cells or by monoclonal antibodies (mAbs) raised against natural LT. Furthermore, antisera that recognize the p33/LT

complex and the other polypeptide complexes of this invention block the mixed lymphocyte reaction (MLR), a standard immunological assay of the expected proliferative response of T lymphocytes to allogenic stimulation, i.e., the introduction of T lymphocytes from another individual, which are recognized as foreign (non-self) by the "responder" lymphocytes. [See, e.g., M. Shalaby et al., "The Involvement of Human Tumor Necrosis Factors-α And -ß In the Mixed Lymphocyte Reaction", J. Immun., 141, 499 (1988)].

When sequenced, the LT protein from the p33/LT complex isolated from cells yields a sequence exactly matching that described for secreted lymphotoxin, i.e., (SEQ ID NO:1) Leu Pro Gly Val Gly Leu Thr Pro Ser [P. Gray et al., "Cloning and Expression of cDNA For Human Lymphotoxin, A Lymphokine With Tumor Necrosis Activity", Nature, 312, pp. 121-124 (1984)]. However, the p33/LT complex and the other polypeptide complexes of this invention may comprise human or animal lymphotoxin, recombinant lymphotoxin, soluble lymphotoxin, secreted lymphotoxin and active fragments of them.

The N-terminal portion of the p33 protein and the related polypeptides of this invention contain the amino acid sequence (SEQ ID NO:2) Gly Leu Glu Gly Arg Gly Gln Arg Leu Gln or (SEQ ID NO:3) Gly Leu Glu Gly Arg Leu Gln Arg Leu Gln. The sequence at the 6th cycle appeared to be a mixture of both G and L indicating possible polymorphism. The p33 protein and the other polypeptides of this invention may have one of these sequences or both.

We believe that the polypeptide complexes of this invention are important in T cell activation events and are useful in compositions and methods for T cell activation or T cell suppression and as

therapeutic agents in the treatment of inflammation or applications requiring cytolytic activities, such as inhibition of tumor cell or neoplasia growth. We also believe that the polypeptide complexes may be important in cellular immunotherapies, including enhancing the tumoricidal properties of tumor infiltrating lymphocytes in Tumor Infiltrating Lymphocyte ("TIL") therapy. Antibodies to p33, its related polypeptides, the p33/LT complex or the other polypeptide complexes of this invention may also disrupt critical LT interactions with particular receptors, thus specifically affecting LT-mediated events other than those mediated through the known TNF receptor forms. Likewise, receptors for TNF, LT or P33/LT, or their derivatives (e.g., soluble receptor immunoadhesins), may be used to inhibit the polypeptides and complexes of this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts flow cytofluorometric analysis of OKT3-stimulated, IL-2 expanded peripheral blood lymphocytes (PBL) showing reaction with 3 different rabbit anti-rLT antisera and showing essentially no reaction with rabbit anti-rTNF antisera.

Figure 2 depicts flow cytofluorometric analysis of a human T cell hybridoma, II-23.D7, showing the presence (following PMA treatment) of a LT-related epitope on the cell surface.

Figure 3 depicts the ability of PMA activated II-23.D7 cells to bind anti-rLT antibodies. Samples of anti-rLT antisera were incubated with PMA-treated U937 (non-LT-producing) cells, (-O-), PMA-activated II-23.D7 hybridoma cells (10^8, -■- ; 10^7, -•-), and no cells (control, -□-). Serial dilutions of the cell-free antisera after incubation were added to rLT and used in

a cytotoxicity assay against L929 (LT-sensitive) cells. The plots indicate that rLT neutralizing antibodies were removed from the antisera by the activated II-23.D7 cells.

Figure 4 shows two autoradiographs (A and B) depicting immunoprecipitation of ^{125}I-labeled surface proteins from PMA-activated II-23.D7 cells. Figure 4A shows immunoprecipitation of an approximately 25 kD surface protein (LT) and an approximately 33 kD surface protein (p33) by post-immune rabbit anti-rLT antiserum but not by pre-immune rabbit serum. Figure 4B shows a 1-dimensional CNBr cleavage map of the 25 kD and 33 kD bands from figure 4A, compared against recombinant TNF (rTNF) produced in E.coli, and recombinant lymphotoxin (rLT) produced in CHO cells, [J. Browning et al., "Studies Of The Differing Effects of Tumor Necrosis Factor And Lymphotoxin On The Growth Of Several Human Tumor Lines", J. Immun., 143, 1859 (1989)] both with (+) and without (-) CNBr cleavage.

Figure 5 presents autoradiographs showing immunoprecipitation of TNF- and LT-related proteins from PMA-stimulated II-23.D7 cells metabolically labeled with ^{35}S-methionine or ^{35}S-cysteine. The figure shows recognition by anti-rLT antisera (L), but not preimmune (P) or anti-rTNF antisera (T), of an approximately 25 kD methionine-containing surface protein (LT) and an approximately 33 kD methionine- and cysteine-containing surface protein (p33). The autoradiographs also indicate that activated II-23.D7 cells also produce a 26 kD form of TNF and secrete soluble lymphotoxin.

Figure 6 depicts affinity purification 1-D CNBr peptide mapping of those proteins from PMA-activated II-23.D7 cells recognized by anti-rLT serum. Figure 6A represents SDS PAGE analysis of the proteins

eluted from an anti-LT affinity column prepared from either pre-immune (PRE) or post-immune (POST) rabbit sera. Figure 6A shows the ~33kD and ~20kD protein bands did not bind to an affinity column prepared using preimmune serum (PRE) but did bind to an affinity column prepared using anti-rLT antiserum (POST). Figure 6B shows partial CNBr cleavage of the ~33 kD and ~20 kD proteins eluted from the POST column, compared against rTNF and rLT run in parallel. The gels were visualized by silver staining.

Figure 7 presents autoradiographs of the ~25 kD and ~33 kD ^{125}I-labeled proteins (designated "sLT" and p33, respectively) immunoprecipitated from activated II-23.D7 cells, treated with N-glycanase (N-gly), with a mixture of neuraminidase and O-glycanase (O-gly), or with all three enzymes.

Figure 8 depicts the results of a reimmunoprecipitation of the coprecipitated p33 and LT proteins to further investigate whether they are immunogenically related.

Figure 9 (comprising parts 9A and 9B) shows the results of isoelectric focusing under denaturing conditions of the immunoprecipitated p33 and p25 (LT) proteins.

Figure 10 (comprising parts 10A and 10B) shows the results of isoelectric focusing under native conditions of the immunoprecipitated p33 and p25 (LT) proteins. Together Figs. 9 and 10 indicate that p33 and LT form a denaturable complex.

Figure 11 depicts flow cytofluorometric analysis of surface proteins differentially expressed on T cells and monocytes after stimulation with a mixture of LPS, IFN-γ and OKT3. From a stimulated PBL pool, separated T cells were observed to express a surface protein recognized by anti-rLT antisera (LT),

whereas separated monocytes expressed a surface protein recognized by anti-rTNF antisera (TNF).

Figure 12 shows flow cytofluorometric analysis of surface LT forms on leu-19$^-$ and leu-19$^+$ (i.e., natural killer) cells treated with IL-2. Analysis of IL-2 treated PBL with both labeled leu-19 and anti-rLT confirms that lymphokine-activated killer (LAK) cells express a surface LT form.

DETAILED DESCRIPTION OF THE INVENTION

In order that the invention herein described may be fully understood, the following detailed description is set forth.

This invention relates to polypeptides that comprise an amino acid sequence selected from the group of (SEQ ID NO:2) Gly Leu Glu Gly Arg Gly Gln Arg Leu Gln and (SEQ ID NO:3) Gly Leu Glu Gly Arg Leu Gln Arg Leu Gln. Preferably, they also have a molecular weight of 31 to 35 kd. Most preferably they are p33. The polypeptides of this invention may be associated with a cell surface or not associated with such surface.

The polypeptide complexes of this invention comprise a polypeptide comprising an amino acid sequence selected from the group of (SEQ ID NO:2) Gly Leu Glu Gly Arg Gly Gln Arg Leu Gln and (SEQ ID NO:3) Gly Leu Glu Gly Arg Leu Gln Arg Leu Gln and a lymphotoxin. More preferably the complex is p33/LT. These complexes may be cell associated or not associated with a cell.

The polypeptide complexes of this invention are recognized by polyspecific antisera raised against recombinant lymphotoxin (rLT) expressed in transfected Chinese Hamster Ovary (CHO) cells and a commercial anti-LT monoclonal antibody (Boehringer Mannheim), indicating that the complex exhibits LT epitopes. In

addition, since the same monoclonal antisera that recognizes the complexes also blocks the mixed lymphocyte reaction (MLR), but monoclonal anti-rLT antibodies do not block the MLR, the complexes of the present invention appear to play an important role in T cell activation. We also expect these complexes to have T cell regulatory activities and cytotoxic activities similar to those of soluble LT or TNF.

This invention also relates to DNA consisting essentially of a DNA sequence encoding on expression a polypeptide comprising the amino acid sequences described above, recombinant DNA molecules characterized by that DNA, hosts selected from the group of unicellular hosts or animal and human cells in culture transfected with that DNA, and recombinant methods of using that DNA and those recombinant DNA molecules and hosts to produce the polypeptides coded thereby.

Flow Cytofluorometric Analysis

First we demonstrated the expression of LT-related epitopes on the surface of T cells using flow cytofluorometric analysis. We observed that human peripheral blood mononuclear cells activated with OKT3 monoclonal antibody demonstrated expression of LT-related epitopes by reacting with anti-rLT antisera. Only anti-rLT antisera, not anti-rTNF antisera, bound to OKT3-stimulated primary T cells.

We also observed that a human T cell hybridoma, II-23.D7 [C. Ware et al., "Human T Cell Hybridomas Producing Cytotoxic Lymphokines: Induction of Lymphotoxin Release And Killer Cell Activity By Anti-CD3 Monoclonal Antibody Or Lectins And Phorbol Ester", Lymph. Res., 5, 313 (1986)], secreted LT upon PMA stimulation and also expressed surface LT-related

epitopes upon PMA stimulation. We also demonstrated that PMA-activated II-23.D7 cells were able to remove LT-neutralizing antibodies from the rabbit anti-rLT antisera, while control U937 cells, which lack all surface LT forms, were not. We further ruled out the possibility that the rabbit anti-rLT antisera had bound (complexed) with rabbit LT (the resulting complexes subsequently binding to cellular LT/TNF receptors on the II-23.D7 cells) by saturating the cellular receptors with excess soluble TNF or LT and observing that this had no effect on the staining. These assays demonstrate that the LT-related epitopes on this hybridoma are genuinely related to LT.

We also observed that pretreatment of the antisera with excess rLT blocked the ability of the antisera to stain II-23.D7 cells, while pretreatment with rTNF had no effect. This assay demonstrated the specificity of the antisera for lymphotoxin-related epitopes.

Trypsinization of the stimulated II-23.D7 cells prior to staining led to loss of the signal, demonstrating that the epitopes recognized by the antisera were proteins.

We also demonstrated that CHO-derived contaminants did not contribute to the antisera recognition of induced proteins on the surface of activated II-23.D7 cells by showing that CHO cells stably transfected with the LT gene, which produce only soluble LT, were not stained by the anti-LT antisera.

Immunoprecipitation

We further characterized these surface LT-related proteins by either surface iodination (^{125}I-labelling) or metabolic labelling (^{35}S-Met or ^{35}S-Cys) of PMA-activated II-23.D7 cells, followed by

solubilization of the plasma membrane with detergent and immunoprecipitation of the labeled LT-related proteins.

Surface iodination coupled with immunoprecipitation revealed two proteins recognized by the anti-LT antisera: a 25-26 kD form subsequently referred to as LT, and a 31-35 kD form subsequently referred to as p33. We observed that neither the preimmune serum from the same rabbit nor anti-rTNF rabbit serum immunoprecipitated these bands from the iodinated, PMA-activated II-23.D7 cells. One dimensional partial CNBr peptide mapping of the iodinated, immunoprecipitated bands showed that the 25-26 kD form (LT) cleaves in a pattern identical to that of iodinated recombinant LT, reinforcing the correlation between LT and soluble LT. The iodinated 31-35 kD form (p33) was not cleaved by CNBr, indicating that it is distinct from the known LT gene product.

We further characterized the LT and p33 proteins by metabolic labelling of PMA-activated II-23.D7 cells with ^{35}S-methionine or ^{35}S-cysteine, followed by immunoprecipitation. The distribution of cysteine and methionine provides a means of distinguishing between TNF and LT and between forms of each with and without their signal sequences [M. Kriegler et al., "A Novel Form Of TNF/Cachectin Is A Cell Surface Cytotoxin Transmembrane Protein: Ramifications For the Complex Physiology Of TNF", Cell, 53, 45 (1988)]. Secreted TNF contains cysteine, but not methionine, while secreted LT contains only methionine and no cysteine residues. LT, however, has one cysteine residue in its signal sequence, while TNF contains two methionine residues in its signal sequence.

We labeled separate cultures of PMA-treated II-23.D7 cells with either ^{35}S-methionine or ^{35}S-cysteine and precipitated immunoreactive proteins from the culture media and the cells. Subsequent SDS-PAGE analysis of the immunoprecipitates from the culture media of cells labeled with ^{35}S-methionine revealed a 25 kD form of LT while the immunoprecipitates from the culture media of cells labeled with ^{35}S-cysteine did not, a pattern expected for secreted LT. Analysis of the washed cells showed both the 25-26 kD LT form and the 33 kD p33 form. These results parallelled the membrane-associated forms observed using surface iodination.

The 25-26 kD LT lacked cysteine, indicating processing of the leader sequence. We also observed that the 33 kD p33 incorporated both ^{35}S-methionine and ^{35}S-cysteine, distinguishing itself as different from the 25 kD LT form. Typically, LT bound to its receptor can be cross-linked to the receptor using a chemical linker such as BOSCOES (i.e., (bis [2-[succinimidooxy-carbonyloxy]ethyl] sulfone; Pierce, Rockford, IL). [J.S. Andrews et al., "Characterization of the Receptor for Tumor-Necrosis Factor (TNF) and Lymphotoxin (LT) on Human T Lymphocytes", J. Immun., 144, 2582 (1990)]. We observed that when surface iodinated II-23.D7 cells were treated with a cross-linking agent, there was no association of either the 25-26 kD LT or the 33 kD p33 related form with an additional membrane protein. This assay demonstrated that receptor binding is not the mechanism by which LT and p33 remain associated with the cell membrane. Furthermore, partial sequence analysis of p33 showed no relationship to either of the two TNF-receptor forms [C. Smith et al., Science, 248, 1019 (1990); T. J. Schall et al., Cell, 61, 361 (1990)].

Affinity Chromatography

Further characterization of the p33 and LT proteins on the surface of II-23.D7 cells was obtained through affinity chromatography. We observed that p33 and LT on the surface of PMA-treated II-23.D7 cells bound to lentil lectin, indicating a glycoprotein structure for each form. Hence a lentil lectin chromatography step was used as a purification step prior to antisera affinity chromatography. We bound detergent-solubilized PMA-treated II-23.D7 proteins to lentil lectin sepharose and eluted with α-methyl mannoside. We prepared both control IgG and anti-LT-IgG affinity columns to accurately assess those proteins specifically recognized by the anti-LT antiserum. We then applied the proteins that bound to lentil lectin to these columns. We observed that low pH elution of the columns led to the release of the p33 and LT proteins from the anti-LT affinity column. SDS-PAGE analysis of the eluate closely resembled the SDS-PAGE analysis of immunoprecipitated proteins from surface iodinated PMA-treated II-23.D7 cells. This comparison demonstrated that similar proteins were purified by the two methods.

We observed that during affinity purification, the ~25 kD LT form appeared to be cleaved to a 19-20 kD form, or a "des 20" form. The original isolation of natural LT from the RPMI 1788 tumor cell line [B. Aggarwal et al., "Primary Structure of Human Lymphotoxin Derived From 1788 Lymphoblastoid Cell Line", J. Biol. Chem., 260, pp. 2334-2344 (1985)] also yielded an N-terminally cleaved 20 kD LT form. One of the methionines is lost in this "des-20" natural LT form, producing a different CNBr cleavage pattern from the intact molecule. One-dimensional CNBr digests of

the affinity-purified LT protein demonstrated a cleavage pattern that is consistent with the truncated natural LT form, and we concluded that the affinity-purified "des-20" LT form probably results from a similar cleavage as observed with the natural LT "des-20" form.

We further observed that p33 generates a doublet upon partial CNBr cleavage. The cleavage pattern generated by the p33 protein demonstrated that one methionine residue was present, and the involved methionine was within 5-20 residues from either the C- or N-terminus. This pattern suggested that p33 does not contain the entire known LT sequence.

We observed that the p33 protein is also expressed by antigen-activated primary cytotoxic T lymphocyte clones. Metabolic labelling of these cells followed by immunoprecipitation with anti-rLT revealed p33 along with small amounts of LT. These results demonstrated that p33 is made by primary T cells as well as by the II-23.D7 hybridoma.

Initial purification of the p33 and LT proteins

We purified these LT and p33 proteins using the following general steps. We first added phorbol myristic acetate (PMA) to II-23.D7 cells. After 24 hours we harvested the cells and washed them with cold serum-free RPMI medium. To the chilled cell pellet we added ice-cold lysis buffer (HEPES, NP-40, EDTA, NaCl, and sodium azide) to which benzamidine, phenyl methyl sulfonyl chloride (PMSF), and N-ethyl maleimide (NEM), soybean trypsin inhibitor, pepstatin and aprotinin had been freshly added. We homogenized the cells gently in a Dounce homogenizer and centrifuged the lysate. We centrifuged and collected the supernatant. We loaded the supernatant onto a lentil-lectin sepharose column

equilibrated in lysis buffer to which we had added $CaCl_2$ and $MnCl_2$. We washed the column with lysis buffer with $CaCl_2$ and $MnCl_2$ and then eluted with lysis buffer containing α-methyl mannoside. We pooled the eluate fractions and loaded directly onto a rabbit nonspecific IgG sepharose affinity column which was directly connected to a rabbit anti-rLT sepharose affinity column. We washed both columns with the same lysis buffer with EDTA followed by lysis buffer wherein the NP-40 had been replaced with MEGA-8 (Octanoyl-N-methyl glucamide, Boehringer-Mannheim). We eluted the washed columns individually with a solution of MEGA-8, glycine, NaCl, benzamidine, and EDTA. The first fractions following the pH shift were pooled, lyophilized and resuspended in water with SDS, and dialyzed against a solution of HEPES and SDS. We dried the dialyzed fractions on a speed-vac and resuspended in water. We mixed aliquots with Laemmli loading buffer and electrophoresed on SDS-PAGE. We visualized proteins by silver staining.

We observed that LT epitope(s) are present on the surface of the II-23.D7 T cell hybridoma only following cell activation such as occurs with PMA treatment. In contrast, when present on primary T-cells, PMA treatment leads to loss of the surface antigen. Additionally, we found that polyclonal antisera to recombinant LT (produced in CHO cells) and natural LT (e.g., Genzyme, Boston, Mass.) rabbit anti-human lymphotoxin antiserum recognized the LT epitope(s).

We have also observed that our antisera recognizing p33 and LT blocks the MLR, whereas a particular monoclonal antibody recognizing a soluble LT does not [M. Shalaby et al., "The Involvement of Human Tumor Necrosis Factors-α And-ß In the Mixed Lymphocyte

Reaction", J. Immun., 141, 499 (1988)]. The p33/LT complex of the invention, therefore, may be a mediator in T cell activation.

The presence of p33 with LT in immunoprecipitates from cell lysates suggested that either p33 is antigenically related to LT or that p33 is bound to LT or both. To address this issue 25 kD and 33 kD bands from ^{35}S-methionine labeled cells were immunoprecipitated with rabbit polyclonal anti-rLT serum, eluted from excised gel slices and subjected to reimmunoprecipitation with either anti-rLT polyclonal serum or mAb. LT, but not p33, could be immunoprecipitated with either anti-rLT antibodies suggesting that p33 is not antigenically related to LT. These observations indicated that p33 is physically associated with LT.

To further investigate the hypothesis that surface LT and p33 form a complex, we performed isoelectric focusing (IEF) experiments under both denaturing and native conditions, the rationale being that if LT and p33 are physically associated, then they should focus as a complex under native conditions but as separate entities under denaturing conditions. The individual isoelectric points (pI's) for LT and p33 were determined by two-dimensional gel analysis (denaturing conditions) (Fig. 9A). LT (p25) possesses five charged isomers ranging in pI from 6.5 to 7.3, whereas p33 possesses four charged isomers ranging in pI from 5.5 to 6.0. When focusing was performed under native conditions, however, LT and p33 focused together as a broad band ranging in pI from 6.3 to 7.2 (Fig. 10A, lanes 6-8). Therefore, the migration of p33 was significantly retarded under native conditions.

Further purification and identification of LT and p33

We later purified these LT and p33 proteins using the following general steps. We grew II-23.D7 cells in RPMI medium with fetal bovine serum and we harvested the cells from 50 l of RPMI and resuspended them in medium and we added phorbol myristoyl acetate (PMA). After activation for 6 hours, we harvested the cells by centrifugation and washed them with Dulbecco's phosphate buffered saline. We suspended the final cell pellet in cold lysis buffer and passed the pellet once through a nitrogen cavitator. We centrifuged the lysed cells and discarded the supernatant. We extracted the pellet overnight in lysis buffer with detergent and then centrifuged it again.

We added the supernatant containing the detergent solubilized membranes to affinity resin composed of monoclonal anti-lymphotoxin coupled to Affi-gel (10) and rocked the suspension overnight. We collected the resin into a small column and washed it with HEPES with nonidet P40, and then with the same buffer with 1% w/v MEGA-8; we eluted the bound proteins with MEGA-8 in glycine buffer and neutralized the fractions immediately with Tris base. We determined the presence of p33 and LT in the fractions by SDS-PAGE analysis and silver staining. We pooled fractions containing these proteins and added SDS, and we dialyzed the pool against 0.1x laemmli sample buffer (using multiple changes to remove the MEGA-8 detergent). We lyophilized the dialyzed solution to dryness and resuspended it in 1/10th the original volume of water. We ran the sample on an SDS-PAGE gel, blotted onto a ProBlot membrane and stained with Coomassie blue dye. We excised the p33 and 25 kD LT bands and loaded them into a protein sequencer. We obtained the N-terminal sequence by Edman degradation

with model 470 A Applied Biosystems sequencer coupled to a 120 PTH amino acid analyzer. We found the sequence of the membrane associated lymphotoxin band to exactly match that described for secreted lymphotoxin, i.e., (SEQ ID NO:1) Leu Pro Gly Val Gly Leu Thr Pro Ser [P. Gray et al., "Cloning and Expression of cDNA For Human Lymphotoxin, A Lymphokine With Tumor Necrosis Activity", Nature, 312, pp. 121-124 (1984)]. The N-terminal portion of the associated p33 protein included the amino acid sequence(s): (SEQ ID NO:2) Gly Leu Glu Gly Arg Gly Gln Arg Leu Gln or (SEQ ID NO:3) Gly Leu Glu Gly Arg Leu Gln Arg Leu Gln. The sequence at the 6th cycle appeared to be a mixture of both glycine and leucine indicating possible polymorphism. The p33 protein may have one of these sequences or both.

In each case where a surface LT form was detected, we were also able to detect p33 (i.e., in PMA-activated II-23.D7, activated CTL clones, and Hut-78 cells constitutively expressing a surface LT form). Because LT is secreted from transfected CHO cells and from B lymphoblastoid cell lines in the absence of a surface LT form, and because the presence of p33 is associated with surface-bound LT, we concluded that p33 complexes with LT to target it to the cell surface. Biochemically, p33 and LT co-migrate on a non-denaturing isoelectric focusing gel, but when the complex is dissociated with urea, the two proteins run separately. [See Figs. 9A, 10A.] These observations have led us to conclude that LT and p33 exist as a complex on the cell surface.

Potential uses of p33 and LT and the p33/LT complex

Given the apparent equivalence between soluble LT and the LT of the preferred p33/LT complex of this invention, the complexes of this invention are

expected to have a number of potential uses including anti-tumor, T cell activating, or T cell suppressing applications, as well as uses in anti-inflammatory compositions and methods. Highly purified p33 will also be useful in synthesizing probes which, in turn, are useful to isolate DNA coding for the p33 and the other proteins of this invention. Such DNA sequences, recombinant DNA molecules including them, and unicellular hosts, and animal or human cells in culture, transfected with them may then be employed to produce large amounts of the polypeptides of this invention, substantially free from other human proteins, for use in the compositions and therapies noted above.

More specifically, we can use our purified p33 protein to determine the amino acid sequences of various portions and fragments. We then use those sequences and the degenerate DNA sequences coding for them to design a series of DNA probes potentially useful in screening various DNA libraries for DNA sequences coding for the p33 and other proteins of this invention. Such libraries include chromosomal gene banks and cDNA or DNA libraries prepared from tissue or cell lines that are demonstrated to produce the p33 protein of this invention. Such cell lines include cell lines well known in the art.

Lymphocytes expressing on their surfaces the polypeptides complexes of this invention, and preferably a p33/LT complex, represent a subset that may have enhanced abilities to kill tumor cells. As such, this subset would be useful in LAK (lymphokine-activated killer) cell or TIL (Tumor Infiltrating Lymphocyte) cell therapies. [H. Thomas, K. Sikora, "Biological Approaches to Cancer Therapy", Jour. Int. Med. Res., 17, 191 (1989)].

Antibodies or antibody derivatives to the polypeptides and polypeptide complexes of this invention are also useful in conventional immunological methods, e.g., panning or flow cytofluorometric sorting, to enrich for this cell population. [L.J. Wysocki and U.L. Sato, "Panning for Lymphocytes: A method for Cell Selection", Proc. Natl. Acad. Sci. USA, 75, 2844 (1978)].

It is also contemplated that the polypeptides and complexes of this invention, or fragments or derivatives thereof, will be useful in cell regulatory or therapeutic applications similar to those in which lymphotoxin and tumor necrosis factors are used.

Administration of the the complexes and polypeptides of this invention, or perhaps peptides derived or synthesized therefrom or using their amino acid sequences, or their salts or pharmaceutically acceptable derivatives thereof, may be via any of the conventionally accepted modes of administration of agents which exhibit anti-tumor, T cell-activating, T cell-suppressing or anti-inflammatory activity. These include oral, parenteral, subcutaneous, intravenous, intralesional or topical administration. Furthermore, the sequence information provided herein will enable the isolation and sequencing of cDNA coding for p33, and recombinant genes for p33 and related polypeptides based thereon will be useful therapeutically, for example in TIL therapy, where a p33 gene, either with or without an LT gene, is introduced into T cells isolated from a tumor and introduced to the patient. More preferably, the cells are taken from the patient, transfected with a DNA sequence encoding on expression a polypeptide of this invention, before or after that transfection incubated with a lymphokine, preferably IL-2, and returned to the patient. The transfected T

cells (now expressing p33 and also consequently complexing LT) home in on the tumors from which they were removed, where the tumorcidal action of LT is delivered directly to the tumors. Likewise, it is contemplated that a p33 gene introduced into and transfecting LAK cells would increase the number of surface complexes on the cells and enhance their activity.

In general, LT and TNF exhibit qualitatively the same spectra of activities, and LT and TNF are believed to interact with the same set of receptors (designated the 55 and 80 kD TNF receptors: C. Smith et al., Science, 248, 1019 (1990); T. Schall et al., Cell, 61, 361 (1990). Nonetheless, the quantitative patterns of biological potency exhibited by LT and TNF are dramatically different, with LT often being much less potent than TNF (see, e.g., Browning and Ribolini, J. Immunol., supra). These observations are difficult to reconcile with the existing receptor binding data. It is possible that the p33/LT complex imparts unique properties on LT such that it now interacts with other as yet undefined receptors. In this case, a p33/LT complex and the other complexes of this invention would have unique biological properties distinguishing them from either LT or TNF. The p33/LT complex may be used to identify and clone such p33/LT or p33 specific receptors. Moreover, further use of the complex may reveal novel biological activities.

Also, while a number of T cell and macrophage cell lines are known to be infectable by the HIV virus, in practice only a small number of cell lines have been useful in propagating the virus in tissue culture. For example, the H9 line, a derivative of Hut-78 originally exploited by Gallo et al. [M. Popovic et al., Science, 224, 497-500 (1984)], and another human lymphocytic

line, C8166, have been valuable for HIV propagation [M. Somasundaran and H. Robinson, Science, 242, 1554-1557 (1988)]. It is possible that surface LT expression or the capacity for expression of surface LT makes a given cell a good target for HIV proliferation.

A role for TNF has been proposed in enhancing HIV proliferation [L. Osborn et al., Proc. Natl. Acad. Sci. USA, 86, 2336 (1989); Z. Rosenberg and A. Fauci, Immunol. Today, 11, 176 (1990); C. Locardi et al., J. Virology, 64, 5874 (1990); G. Oli et al., Proc. Natl. Acad. Sci. USA, 87, 782 (1990)]. We have found that the II-23.D7 line is infectable with the HIV strain IIIB, but upon PMA treatment the infection by the virus is dramatically increased. The Hut-78 cell line was found to constitutively express a surface LT form, and the C8166 line resembles II-23.D7 in that surface LT appears following PMA treatment. Considering these results on the infectability of II-23.D7 by HIV and the relationship between infectable cell lines and surface LT expression, we propose that those lines may be good hosts for HIV infection and replication because the p33/LT complex and the other polypeptides and complexes of this invention serve a regulatory role. It has been demonstrated that the LT gene is induced by expression of the HIV transcriptional activator TAT [K. Sastry et al., J. Biol. Chem., 265, 20091 (1990)] and, moreover, HTLV-1 infection has also been shown to induce LT expression [N. Paul, et al., J. Virol., 64, 5412 (1990); E. Tschachler et al., in Human Retrovirology (Raven Press 1990), W. Blattner, eds., p. 105]. Thus, induction of LT by HIV infection and consequent p33/LT complex or other complex expression, or induction of p33/LT or other complex expression by PMA treatment in cell lines competent to make these proteins, may serve to enhance viral replication. For

this reason, antibodies or specific binding proteins (e.g., soluble receptors) to the p33/LT complex or the other polypeptide complexes of this invention or to soluble forms of those complexes or to p33 and the other polypeptides of this invention may at least partially inhibit or block HIV proliferation.

The compositions used in these therapies may also be in a variety of forms. These include, for example, solid, semi-solid and liquid dosage forms, such as tablets, pills, powders, liquid solutions or suspensions, suppositories, injectable and infusible solutions, genetic therapy. The preferred form depends on the intended mode of administration and therapeutic application. The compositions also will preferably include conventional pharmaceutically acceptable carriers and may include other medicinal agents, carriers, genetic carriers, e.g., Tumor Infiltrating Lymphocyte therapy, adjuvants, excipients, etc., e.g., human serum albumin or plasma preparations. Preferably, the compositions are in the form of a unit dose and will usually be administered one or more times a day.

The composition of this invention will be administered at an effective dose to treat the particular clinical condition addressed. Determination of the particular dose for a given application is well within the skill of the art taking into consideration, for example, the condition and weight of the patient, the extent of desired treatment and the tolerance of the patient for the treatment.

The following are examples which illustrate the p33/LT complex of this invention and the methods used to characterize it. These examples should not be construed as limiting.

EXAMPLES

We used the following experimental procedures in the examples:

Antisera

Recombinant human LT (rLT) was expressed and secreted by a stably transfected chinese hamster ovary (CHO) cell line into serum-free conditioned media. We purified the secreted rLT from the serum-free conditioned media by a series of Sepharose S, lentil lectin-sepharose and FPLC Mono Q column chromatography steps. The properties of the CHO cell-derived rLT preparation have been described. [J. Browning et al., "Studies On The Differing Effects Of Tumor Necrosis Factor And Lymphotoxin On The Growth Of Several Human Tumor Lines", J. Immun., 143, 1859 (1989)]. We immunized two rabbits (4 and 5) by a lymph node procedure [M. Sigel et al., "Production Of Antibodies By Inoculation Into Lymph Nodes", Met. Enz., 93, 3 (1983)] with 25 μg of native rLT in complete Freund's adjuvant. A third rabbit (6) was immunized via the same route with 25 μg of denatured rLT in complete Freund's adjuvant. We prepared denatured rLT by SDS-PAGE followed by electroelution into 0.1% SDS-carbonate buffer.

Using the above methods, three anti-rLT antisera were generated, two directed against native rLT and a third against SDS-denatured rLT. The antisera raised by immunization with native protein (rabbits 4 and 5) could neutralize a 50 unit/ml solution at a dilution of 1:2000-5000. The serum raised against denatured rLT (rabbit 6) lacked neutralizing titer, but was weakly reactive with rLT on a Western blot. None of the antisera could neutralize r-human TNF nor could they recognize r-human TNF bound

to an ELISA plate except for a very weak titer in the antiserum from rabbit 6. Only antiserum from rabbit 6 was capable of recognizing rLT in a western analysis.

We immunized a fourth rabbit with recombinant human TNF. We prepared the polyclonal anti-rTNF rabbit serum via a classical immunization scheme using recombinant human TNF (E.coli derived [D. Weir et al., Handbook Of Experimental Immunology In Four Volumes, Chapter 8 "Immunization Of Experimental Animals"]) in complete Freund's adjuvant followed by a boost in incomplete Freund's adjuvant. The serum raised against rTNF by immunization had a good neutralizing titer. A neutralizing monoclonal antibody to TNF has been described [Liang et al., "Production And Characterization Of Monoclonal Antibodies Against Recombinant Human Tumor Necrosis Factor/Cachectin", Biochem. Biophys. Res. Comm., 137, 847 (1986)]. Pre-immunization serum was collected from all animals for use as controls.

Cell Growth and T cell Activation

All cells were obtained from the American Type Culture Collection (ATCC), except for the LT transfected Chinese hamster ovary (CHO) line that was described previously [Browning and Ribolini, "Studies on the Differing Effects Of Tumor Necrosis Factor And Lymphotoxin On The Growth Of Several Human Tumor Lines", J. Immun., 143, pp. 1859-1867 (1989)].

Cells were grown in RPMI 1640 supplemented with 1% glutamine, 10 mM HEPES buffer, pH 7.5, penicillin/streptomycin and 10% fetal bovine serum (Hyclone-defined) (designated "complete RPMI"), except for the transfected CHO cells which were grown in Dulbecco's modified Eagle's medium supplemented as above. The human T cell hybridoma, II-23, was a result

of a fusion of the human CEM tumor line with activated peripheral T lymphocytes and was further subcloned (II-23.D7) [C. Ware et al., "Human T Cell Hybridomas Producing Cytotoxic Lymphokines: Induction of Lymphotoxin Release And Killer Cell Activity By Anti-CD3 Monoclonal Antibody Or Lectins And Phorbol Ester", Lymph. Res., 5, 313 (1986)]. Human peripheral blood lymphocytes (PBL) were drawn into heparinized glass tubes, isolated by Ficoll-Hypaque centrifugation, washed and resuspended in complete RPMI medium. We treated PBL at 2 x 10^6 cells/ml with a 1:500 dilution of OKT3-conditioned medium (~2 ng/ml final) in the presence of 1 μg/ml indomethacin and, in some experiments, with 10 ng/ml rIL-2 (Biogen, Inc., Cambridge, MA)). Human CTL-clones were generated as described [L. Green et al., "Cytotoxic Lymphokines Produced By Cloned Human Cytotoxic T Lymphocytes", Jour. of Immun., 135, 4034 (1985)] and activated either with irradiated stimulator cells (antigen) or a combination of anti-CD2 monoclonal antibodies ($T11_2$ + $T11_3$) provided by E. Reinherz.

Flow Cytometry

We resuspended cells in RPMI 1640 medium with 10% fetal bovine serum (FBS), 0.1% sodium azide and 0.1 mg/ml human IgG at 0°C. Following preincubation with the human IgG, we added additional media containing the desired antisera. Typically the cells were incubated with a final dilution of the anti-rLT and anti-rTNF sera of 1:200 for 60-90 min. We washed cells twice with Dulbecco's phosphate buffered saline (PBS) and then incubated them with a 1:500 dilution of fluorescein-labeled goat anti-rabbit IgG (Cappel Durham, N.C.) in the above medium for a minimum of 60 min. Cells were then washed once and either

analyzed directly or, in some cases, analyzed following fixation for 10 min. at 0°C with 0.5% paraformaldehyde. We performed two color analyses as above, except that we added phycoerythrin labeled leu-4, leu-2, leu-M3 or leu-16 or leu-19 (Becton-Dickinson, Mountain View, CA) at the second antibody stage. The comparison of surface-bound LT with IL-2 receptor levels was done with separate single color analyses with fluorescein-labeled anti-IL-2 receptor (CD25) antibody (Becton-Dickinson, Mountain View, Ca.). Analyses were performed with a FACStar instrument (Becton-Dickinson).

Absorption of Neutralizing Anti-rLT Antibodies by Activated II-23.D7 Cells

We stimulated II-23.D7 and U937 premonocytic cells at 1 x 10^6 cells/ml for 8 hours with 10 ng/ml of PMA in complete RPMI medium. We washed the cells (1 x 10^8) three times in medium and aspirated the supernatant to obtain a dry pellet. The cells were then resuspended in 1 ml of medium containing a 1:1000 dilution of anti-rLT serum (from rabbit 4) and incubated on ice for 1.5 hours with mixing. Cells were cleared from the antiserum by centrifugation. We mixed the absorbed antiserum (both pre- and post-immune) with an equal volume (50 μl) of medium containing 15 U/ml of rLT and incubated for 20 minutes at room temperature. The mixtures were diluted serially into medium and added to L929 cells (in 0.1 ml) and incubated a further 24 hours. We assessed cell viability by the MTT assay as described [L. Green et al., "Rapid Colorimetric Assay For Cell Viability: Application To The Quantitation of Cytotoxic And Growth Inhibitory Lymphokines" Jour. Immun. Meth., 70, 257-268 (1984)].

^{35}S-Methionine or ^{35}S-Cysteine
Metabolic Labelling of T cells

We transferred cells into either cysteine-free or methionine-free RPMI 1640 supplemented with penicillin/streptomycin, glutamine, 10 mM HEPES pH 7.5, 10% v/v dialyzed FBS and 2% v/v conventional RPMI (cold carrier addition). We adjusted the cell concentration to 2-3 x 10^5 cells/ml and added ^{35}S-methionine or ^{35}S-cysteine to the appropriate medium to a level of 100-200 μCi/ml. In the case of freshly activated PBL, the supernatants were gently removed, and the cells were centrifuged, resuspended in labelling medium and added back to the original adherent population. Following a 12-18 hour labelling period, we washed and lysed the cells as described below. With the PBL, cells were removed by pipetting and the adherent population partially removed by treatment with 5 mM EDTA in PBS.

Immunoprecipitations

To 0.2-0.5 ml of labeled cell lysate we added 2-4 μl of rabbit serum. The sample was left for 1-2 hours at 4°C. We then added a 60 μl aliquot of a 60-75% suspension of washed Protein A sepharose (Pharmacia, Piscataway, N.J.) and rocked the sample for 6-18 hours at 4°C. We washed the Protein A sepharose pellets 3 times with 1% NP-40 in calcium/magnesium free PBS and resuspended them in 50 μl of Laemmli SDS loading buffer. Typically a single lysate sample was cycled through sequential immunoprecipitations with preimmune anti-rLT serum, anti-rTNF antiserum and finally post immune anti-rLT antiserum. In one set of experiments, we added 5 mM $CaCl_2$ and $MnCl_2$ to the lysate and the lysate was rocked overnight with 75 μl of 75% suspension of washed lentil lectin-sepharose. The sepharose was washed twice with NP-40/PBS and then

eluted with 3 consecutive additions of 75 μl of 1% NP-40/PBS with 0.25 M α-methyl mannoside. We subjected the pooled washes to the immunoprecipitation protocol.

Rabbit anti-rLT Affinity Column

We purified the immunoglobulin fraction from the anti-rLT serum (from rabbit 4) using Protein A sepharose with acid pH elution. The eluted IgG-containing fractions were dialyzed against PBS and concentrated by amicon filtration. The anti-rLT-IgG solution (15 ml of 6 mg/ml) was coupled to 8 ml of Affi-gel 10 resin (Biorad, Richmond, Ca.) as per instructions. We prepared an identical affinity column using nonspecific rabbit IgG (Cappel, Durham, N.C.). Both columns were washed with PBS, 1 M acetate pH 3.0 with 1% NP-40 and finally with lysis buffer lacking protease inhibitors.

Purification of p33 and LT

We grew II-23.D7 cells (15 l) to a density of 5×10^5 cells/ml and added phorbol myristic acetate (PMA) to give a final concentration of 25 ng/ml. After 24 hours, we harvested the cells and washed them into cold serum-free RPMI medium. To the chilled cell pellet containing 7×10^9 cells we added 100 ml of ice-cold lysis buffer (50 mM HEPES pH 7.5, 1% v/v NP-40, 2 mM EDTA, 0.15 M NaCl and 0.1% sodium azide) to which 5 mM benzamidine, 1 mM phenyl methyl sulfonyl chloride (PMSF) and 0.25 mM N-ethyl maleimide (NEM), 10 μg/ml soybean trypsin inhibitor, 0.7 μg/ml pepstatin and 10 μg/ml aprotinin had been freshly added. We gently homogenized the cells in a dounce homogenizer and centrifuged the lysate at 10,000 x g for 10 minutes. We centrifuged the supernatant at 60,000 x g for 90 minutes and collected the supernatant.

To the supernatant from the high speed centrifugation we added 5mM $CaCl_2$ and 5mM $MnCl_2$. The supernatant was then loaded onto a 20 ml lentil-lectin sepharose column (Pharmacia, Piscataway, N.J.) equilibrated in lysis buffer plus $CaCl_2$ and $MnCl_2$. We washed the column with lysis buffer (with $CaCl_2$ and $MnCl_2$) and then eluted the column with lysis buffer containing 0.25 M α-methyl mannoside.

We pooled the lentil lectin eluate fractions to give a volume of 50 ml and loaded them directly onto a 2 ml rabbit nonspecific IgG sepharose affinity column. We connected this column directly to a 2 ml rabbit anti-rLT sepharose affinity column. We washed both columns with the same lysis buffer with EDTA, followed by lysis buffer wherein the 1% NP-40 had been replaced with 1% w/v MEGA-8 (Octanoyl-N-methyl glucamide, Boehringer-Mannheim, Indianapolis, IN.).

We eluted the washed columns individually with 1% MEGA-8, 50 mM glycine pH 2.5, 0.05 M NaCl, 5 mM benzamidine, and 2 mM EDTA. We pooled the first 20 ml following the pH shift, lyophilized the pool and resuspended it in 1 ml of water with 0.05% SDS, and dialyzed it against 10 mM HEPES pH 7.5, 0.05% SDS and 0.1% MEGA-8. We dried the dialyzed fractions on a speed-vac and resuspended them in 0.15 ml of water. We mixed aliquots with Laemmli loading buffer and electrophoresed them on SDS-PAGE. The p33 and LT proteins were visualized by silver staining.

Iodination of II-23.D7 Cell Surface

Either control or PMA-induced II-23.D7 cells were washed extensively in calcium/magnesium-free PBS, treated with 1 mM PMSF and 0.25 mM NEM and then washed twice. To a 12 x 75 mm glass tube that was coated with 50 μg of iodogen (Pierce) we added 0.3 ml of cells (1 x

10^7 total) and 1-2 mCi of ^{125}sodium iodide. Cells were left with periodic swirling for 25 minutes at room temperature, washed 3 times in PBS with 10% FBS and resuspended in lysis buffer as described above. We then removed the nuclei with a 2 minute centrifugation in an Eppendorf centrifuge. We then centrifuged the supernatant an additional 15 minutes. The cleared supernatant was subjected to the immunoprecipitation protocol.

1-Dimensional CNBr Peptide Mapping

We electrophoresed samples on a 12% acrylamide SDS-PAGE Laemmli system gel for a short distance and excised the appropriate gel sections. We soaked the gel slices for 1 hour in 1.0 ml of 0.1 N HCl, 0.2% 2-mercaptoethanol with 15 µl of 700 mg/ml fresh CNBr in 90% formic acid. The slices were then removed and washed for 5 minutes with 0.1 M Tris-Cl pH 8.0, 5 min with 25 mM Tris-Cl pH 8.0 and finally 10 min with 1x Laemmli SDS-PAGE loading buffer. We loaded the slices onto a 15% SDS-PAGE Laemmli gel with a 12% acrylamide stacking gel. We visualized the peptide bands by silver staining or autoradiography of the dried gel.

Reimmunoprecipitation

Reimmunoprecipitation of SDS-PAGE-separated antigens was carried out by excising labeled bands from gels, rehydrating them for 10 minutes in TBS, 0.2% SDS, and then dicing the gel slices into small pieces. The proteins were eluted by incubation in 1 ml TBS, 0.2% SDS for 8 hours at room temperature, with rotation. After elution the gel pieces were removed by centrifugation, and NP-40 was added to the supernatant to a final concentration of 2%. The eluted proteins

were then immunoprecipitated as above, and reanalyzed by SDS-PAGE.

Isoelectric Focusing (IEF)

Two-dimensional IEF was performed essentially as described by P. H. O'Farrell [J. Biol. Chem., 250, 4007-4021 (1975)]. ^{125}I-labeled antigens were immunoprecipitated from II-23.D7 cell extracts, and the immunoprecipitated proteins were eluted by heating at 100°C for 5 minutes in 100 µl O'Farrell sample buffer containing 9.5 M urea. The eluted proteins were then focused (first dimension) on 14 cm x 3 mm tube gels possessing a 2% final concentration of ampholines (range pH 3-10, Sigma) at room temperature for 16 hours at a constant voltage (400 V). The second dimension was 12% SDS-PAGE.

For IEF under native (non-denaturing) conditions, ^{125}I-labeled cell extracts were focused directly on tube gels identical to those above except for the presence of urea. The labeled extract (200 µl volume) was centrifuged at 100,000 x g (30 psi, airfuge) for 10 minutes prior to loading onto the tube gel. The focusing was performed at 4°C under the same conditions as described above. The tube gel was then removed and sliced into 1 cm sections, and the proteins were eluted by incubating each slice in 1 ml TBS, 2% NP-40, 2 mM PMSF for 8 hours at room temperature, with rotation. The supernatants containing the eluted proteins were then immunoprecipitated and analyzed by SDS-PAGE. The pH gradients for both the denatured and native tube gels were determined by measuring the pH's of individual slices from gels run in parallel.

T Cell Proliferation Assays

We isolated and resuspended PBL in complete RPMI as described above except for the substitution of

fetal bovine serum with 10% human autologous serum, 1 μg/ml indomethacin and 50 U/ml polymyxin B. In the MLR experiments, autologous serum was the responder's serum. We irradiated stimulator cells from a different donor with 3000 rads. We preheated rabbit sera for 1 hour at 56°C, and diluted and sterile filtered the sera prior to use in proliferation assays. Cells (1×10^5 total) in 0.2 ml in a round bottom 96-well plate were treated with either 5 μg/ml phytohemagglutinin, 1-2 ng/ml OKT3 or $1.5\text{-}2 \times 10^5$ irradiated stimulator cells in the presence or absence of various antisera or cytokines. After 3 days (PHA or OKT3 activation) or 5 days (MLR), cells were pulsed with ^{3}H-thymidine, harvested and counted.

Example 1

T cells Express LT-related Epitopes on Their Surfaces

Under the conditions described above, we activated human peripheral mononuclear cells (PMN) with OKT3 monoclonal antibody and, after two days in culture, we analyzed them for expression of p33/LT complex related forms using flow cytofluorometric analysis. In one experiment, the results of which are shown in Figure 1, we cultured fresh PBL for 3 days with OKT3 and IL-2 and stained them with a 1:200 dilution of antisera to native rLT ("LT-4" and "LT-5" panels on Figure 1, from rabbits 4 and 5 respectively), denatured rLT ("LT-6" panels on Figure 1, from rabbit 6) and native rTNF ("TNF" panel on Figure 1, from rabbit 7). We stained cells with postimmune serum (solid lines on Figure 1 panels) or with preimmune serum from each animal (dotted lines on Figure 1 panels). Figure 1 shows that only anti-rLT sera from

rabbits 4 and 5 recognized epitopes on the activated peripheral T cells.

In the experiment shown in Figure 2, we treated II-23.D7 cells with or without 10 ng/ml PMA for 15 hours and stained them as described in the Figure 1 experiment, with rabbit 4 anti-rLT postimmune serum (solid line on Figure 2 panels) or with rabbit 4 preimmune serum (dotted line on Figure 2 panels). As shown in Figure 2, we found that the T cell hybridoma II-23.D7, which synthesizes LT upon phorbol ester (PMA) stimulation, expressed surface LT-related epitopes upon PMA activation.

To establish that the LT-related epitopes on T cells were related to LT and not to some contaminant in the CHO-cell derived recombinant LT preparation, we treated a 1:1000 dilution of antiserum from rabbit 4 with PMA-activated, washed II-23.D7 or U937 cells. In the experiment shown in Figure 3, we treated a 1 ml sample of anti-rLT sera (1:1000 anti-LT-4) with either no cells (-□-), 1 x 10^8 U937 cells (-O-), 1 x 10^8 (-■-) PMA-activated II-23.D7 cells or 1 x 10^7 (-•-) PMA-activated II-23.D7 cells. We added dilutions of absorbed antisera to a limiting amount of rLT in a L929 cytotoxicity assay such that a 1:4000 final dilution was present in the first well. This assay measures the ability of LT to kill a mouse fibroblast cell line, L929, within a 24 hour period [L. Green, J.L. Reade, C.F. Ware, "Rapid Colorimetric Assay for Cell Viability: Application to the Quantitation of Cytotoxic and Growth Inhibitory Lymphokines", J. Immun. Methods, 70, 257 (1984)]. After 24 hours, we assessed cell viability using a MTT readout. Plotted on Figure 3 is optical density (which is proportional to cell viability) vs. the dilution of absorbed antisera. Data represent the average of duplicate wells and

duplicates generally were within the range defined by the symbol. As shown in Figure 3, analysis of the neutralizing titer of the absorbed antisera in the standard L929 cytotoxicity assay demonstrated that the activated II-23.D7 cells removed the LT neutralizing antibodies, whereas U937 cells were ineffective. These data indicate that the antigenic structures on the membrane surface are actually related to LT.

We subjected the hybridoma II-23.D7 to a number of further treatments to examine a number of trivial explanations for the apparent existence of LT-related epitopes on T cell surfaces. First we ruled out the possibility that LT:antibody complexes in the antisera could bind to TNF/LT receptors on the hybridoma. Both TNF and LT have trimeric structures which could allow for the presence of antibody binding epitopes within the complex. However, prior saturation of the cellular TNF receptors with soluble TNF or LT had no effect on the surface staining. Such saturation should have prevented such an immune complex from binding to such a receptor..

A pH 3 lactic acid treatment, which can release bound TNF from its receptor, had no effect on the signal, suggesting that the LT is not receptor bound. However, experiments utilizing ^{125}I-LT binding to II-23.D7 cells indicated that receptor bound LT was more difficult to remove from its receptor at acidic pH's than TNF.

Mild trypsinization of the cells prior to staining led to a loss of the signal, indicating that the epitope is a protein. To determine whether surface-associated LT was phosphatidylinositol linked, the cells were treated with a phosphatidylinositol specific phospholipase C. Under conditions where a PI-linked antigen, LFA-3, could be released [A. Peterson

et al., "Monoclonal Antibody And Ligand Binding Sites Of The T Cell Erythrocyte Receptor (CD2)", Nature, 329, 842 (1987)], no effect was observed on the LT epitope.

We could not stain CHO cells stably transfected with the LT gene, either with or without prior PMA activation, indicating that antibodies to CHO derived contaminants in the original rLT used to immunize the rabbits were not present in sufficient amounts to contribute to the staining of II-23.D7 cells. Likewise, antibodies generated against any fetal bovine serum proteins contaminating the LT preparation would be ineffective in staining T cells since the staining was performed in 10% fetal calf serum.

Pretreatment of the anti-rLT serum with rLT blocked the staining of LT forms on II-23.D7 cells whereas pretreatment with rTNF did not.

Example 2

Immunoprecipitation of LT-related Proteins on the T cell Hybridoma II-23.D7

We surface iodinated PMA activated II-23.D7 cells and lysed and solubilized the cells in detergent. Immunoprecipitation and SDS-PAGE analysis of the labeled membrane proteins showed that two proteins were recognized by anti-rLT antisera. Figure 4A shows the results of SDS-PAGE analysis of the iodinated surface proteins precipitated with either pre-immune (PRE) or post-immune (POST) anti-rLT serum (from rabbit 4).

As shown in Figure 4A, we observed a 25-26 kD molecular weight form ("LT") that correlated with the expected size of LT, and we also saw an additional form of approximately 33 kD ("p33"). Neither the preimmune serum from the same rabbit (Figure 4A column PRE) nor anti-rTNF rabbit serum were able to immunoprecipitate

any bands from the iodinated, PMA-activated II-23.D7 cells.

1-D partial CNBr peptide mapping of the iodinated bands showed that the 25-26 kD form was cleaved in a pattern identical to that of iodinated rLT, thus identifying this band as LT. In the experiment shown in Figure 4B, the 25-26 kD and 33 kD bands from panel A were excised, subjected to limited CNBr cleavage and electrophoresed on a SDS-PAGE system. For comparison, cleavages of both rTNF and rLT performed in parallel are shown in Figure 4B. The gels were visualized by autoradiography. Lane 1 represents rTNF, lane 2 represents rLT, lane 3 represents LT, and lane 4 represents p33. The increased sizes of the CNBr fragments reflect the increased amount of carbohydrate on natural LT. The iodinated 33 kD form was not cleaved by CNBr (lane 4), indicating that it is different from the known LT gene product. rTNF was not cleaved with CNBr (lane 1) due to the absence of methionine in this protein.

We undertook metabolic labelling with ^{35}S-methionine and ^{35}S-cysteine coupled with immunoprecipitation to characterize further these LT-related surface forms. In the case of the TNF/LT pair, the distribution of cysteine and methionine allows one to distinguish both between TNF and LT and between forms with and without their signal sequences, as was exploited in studies on the membrane TNF form [M. Kriegler et al., "A Novel Form of TNF/Cachectin Is a Cell Surface Cytotoxin Transmembrane Protein: Ramifications For the Complex Physiology of TNF", Cell, 53, pp. 45-53 (1988)]. In the case of the fully processed cytokines, i.e., secreted forms, TNF contains cysteine and not methionine, while lymphotoxin contains only methionine and not cysteine. LT, however, has two

cysteine residues in the signal sequence domain and TNF contains a methionine residue in this N-terminal region. Separate cultures of II-23.D7 hybridoma cells were labeled with either ^{35}S-methionine or ^{35}S-cysteine and immunoreactive proteins were precipitated. In an experiment, the results of which are shown in Figure 5, II-23.D7 cells were activated with 10 ng/ml PMA and simultaneously labeled for 8 hours with either ^{35}S-methionine or ^{35}S-cysteine. Both the medium and lysed cells were subjected to consecutive immunoprecipitations with preimmune (rabbit 4) (P), anti-rTNF (T) and anti-rLT (rabbit 4) (L) sera in that order. Figure 5 shows an SDS-PAGE auto-radiographic analysis of the immunoprecipitates from either supernatants containing secreted proteins or the washed cells. "s-TNF" marks the ^{35}S-methionine labeled anti-rTNF immunoprecipitated band from the cells that was putatively assigned as the unprocessed 26 kD form of TNF. "Met" and "Cys" refer to the ^{35}S-labeled amino acid employed. Those lanes containing ^{35}S-cysteine were exposed for longer periods of time than the lanes containing ^{35}S-methionine. In the supernatants from these cells (lanes labeled "secreted"), a 25 kD form of LT ("LT") was released following PMA treatments by those cells that were labeled with ^{35}S-methionine, but not by those labeled with ^{35}S-cysteine. This pattern is expected for fully processed, secreted LT. Longer exposures showed trace amounts of TNF in the supernatant, and the incorporation of label was as expected for fully processed, secreted TNF. We observed the expression of predominantly LT with low levels of TNF also at the mRNA level (Shamansky and Ware, unpublished observation). Analysis of the washed cells (lanes labeled "cellular") showed that both the 25-26 kD LT, along with the 33 kD p33, were present.

The relative amounts of the 25-26 kD and 33 kD forms paralleled those observed using surface iodination. The 25-26 kD surface LT form lacked cysteine, indicating processing of the leader sequence. The 33 kD form incorporated both ^{35}S-methionine and ^{35}S-cysteine. Longer exposures (not shown) of the film shown in Figure 5 revealed the presence of an anti-TNF immunoprecipitated band from the cells at about 26-27 kD. The band showed incorporation of both labeled cysteine and labeled methionine. The labelling was stronger with cysteine. Since the cys:met ratio is 4:1 in the 26 kD-TNF form, this labelling pattern confirms the identity of this band.

The presence of p33 with LT in immunoprecipitates from cell lysates suggested that either p33 is antigenically related to LT or that p33 is bound to LT or both. To address this issue 25 kD and 33 kD bands from ^{35}S-methionine labeled cells were immunoprecipitated with rabbit polyclonal anti-rLT serum, eluted from excised gel slices and subjected to reimmunoprecipitation with either anti-rLT polyclonal serum or mAb. LT, but not p33, could be immunoprecipitated with either anti-rLT antibodies suggesting that p33 is not antigenically related to LT. These observations indicated that p33 is physically associated with LT. We believe that the 33 kD protein is unrelated antigenically to LT and simply co-precipitated with LT.

With either surface iodination or metabolic labelling, we were unable to detect either of the known 55 or 80 kD TNF/LT receptor forms associated with LT or TNF. Presumably, this is because the receptors are rapidly lost during activation of T cells. [C. Ware et al., "Regulation Of The CTL Lytic Pathway By Tumor Necrosis Factor", Cellular Immunity And The

Immunotherapy Of Cancer, UCLA Symposia on Molecular and Cell Biology M.T. Lotze and O.J. Finn, Eds. Vol. 135, pp. 121-128 (Wiley-Liss, Inc. New York) 1990].

Example 3

Biochemical Characterization of Surface LT-forms on II-23.D7 Cells

We purified the LT-related forms on the surface of PMA-treated II-23.D7 cells using affinity chromatography. Using immunoprecipitation techniques, we had noted that both of p33 and LT bound to lentil lectin sepharose, indicating a glycoprotein structure. We bound detergent solubilized PMA-treated II-23.D7 proteins to lentil lectin sepharose and eluted with α-methyl mannoside prior to affinity purification. We prepared both control IgG and anti-IgG columns to more accurately assess those proteins specifically recognized by the anti-rLT serum. Low pH elution of the columns led to the release of about 100-200 ng of the two LT forms from the anti-rLT column.

Figure 6A reflects SDS PAGE analysis of the proteins eluted from anti-rLT affinity column prepared from either pre-immune (PRE) or post-immune (POST) rabbit sera. In Figure 6B, the 33 kD and 20 kD bands from the gel in panel A were excised and subjected to limiting CNBr cleavage and electrophoresed on a SDS-PAGE system. For comparison, Figure 6B showed CNBr cleavages of rTNF and rLT (CHO-derived) performed in parallel. The gels were visualized by silver staining. SDS-PAGE gels of the eluate resembled closely gels of immunoprecipitated, surface iodinated PMA-treated II-23.D7 cells, indicating that similar proteins had been purified.

During the affinity purification, the 25 kD LT form appeared to be cleaved to a 19-20 kD form,

i.e., it now co-migrated with the intact recombinant CHO cell-derived LT. The original isolation of natural LT from the RPMI 1788 tumor line also yielded an N-terminally cleaved "des-20" LT form. One-dimensional CNBr digests of the affinity purified proteins showed the cleaved 20 kD LT form to have a CNBr cleavage pattern that presumably reflects the truncated nature of this LT form. One of the methionines is lost in the "des-20" LT form and hence the cleavage pattern would be different from that of the intact LT form. The 33 kD protein (p33) generated a doublet upon CNBr cleavage, and from this it was estimated that the single methionine must lie within 5-20 residues from either the C- or N-terminus. This cleavage pattern shows that the 33 kD protein is significantly different from known LT forms. CNBr cleavage analysis of the surface iodinated 33 kD protein probably gave a similar result; however, the resolution achievable with iodination was insufficient to visualize the doublet. Staphylococcus V8 digestion of the iodinated rLT, rTNF and II-23.D7 LT forms showed the rLT and 25-26 kD II-23.D7 LT form to be resistant to digestion, confirming the assignment of this protein as LT. The 33 kD protein was cleaved into several smaller fragments with the pattern resembling closely that of rTNF.

In Figure 7, immunoprecipitated, surface iodinated proteins were resolved on SDS-PAGE analysis and the surface-associated 25-26 kD protein ("sLT") and the 33 kD protein ("p33") bands were excised. Slices were digested with N-glycanase (N-Gly), with a mixture of neuraminidase and O-glycanase (O-Gly), or with all three enzymes. The digested slices were rerun on SDS-PAGE and an autoradiogram of the dried gel is shown. As shown in Figure 7, immunoprecipitation of iodinated

surface LT followed by digestion with either or both N- and O-glycanases showed the 25-26 kD LT form to contain an N-linked oligosaccharide. The 25-26 kD LT form contains only one N-linked site which would correlate well with the size change upon N-glycanase digestion. Likewise, the 33 kD form (p33) lost about 3 kD of size upon treatment with N-glycanase, suggesting the presence of one N-linked oligosaccharide. In contrast to the 25-26 kD LT form, O-glycanase treatment did not affect the molecular weight of p33. The lack of cleavage by a glycanase, however, is not definitive evidence for the lack of a carbohydrate.

Example 4

Reimmunoprecipitation of LT

The coprecipitation of p33 with LT suggested that these proteins are either antigenically related or physically associated. To address this issue we tested whether or not SDS-PAGE separated LT (p25) and p33 proteins could be immunoprecipitated. LT and p33 labeled with ^{125}I or ^{35}S-Met were first partially purified by immunoprecipitation and separated by SDS-PAGE. The labeled bands were excised, rehydrated in buffer, and the proteins eluted. The eluted proteins were then subjected to a second round of immunoprecipitation using either polyclonal or monoclonal anti-rLT antibodies (Fig. 10). Rabbit anti-rLT reimmunoprecipitated LT ("p25", lane 2) but not p33 (lane 3). The anti-rLT mAb precipitated LT (lane 5) and a 21 kD protein ("p21", lane 4), which, as shown below, is a precursor of LT; however, it did not precipitate p33 (lane 6). The results indicate that after LT and p33 are separated by SDS-PAGE, both polyclonal and monoclonal anti-rLT antibodies are capable of reacting with LT but not with p33. This

data provides evidence that p33 is not antigenically related to LT. However, we cannot rule out the possibility that putative p33 cross-reactive epitopes are lost after denaturation, whereas the LT epitopes remain intact.

Figure 8 shows the results of reimmunoprecipitation of ^{125}I-labeled and ^{35}S-Met-labeled p25 and p33 proteins eluted from SDS-PAGE gels. LT and p33 species from ^{125}I-labeled II-23.D7 cells (lanes 2,3) and ^{35}S-labeled cells (lanes 4,5,6) were eluted from gel slices as described above. The elutes were immunoprecipitated with either the anti-rLT serum (lanes 2,3) or the anti-rLT mAb (lanes 4,5,6), and the reprecipitated proteins analyzed by SDS-PAGE and autoradiography. Lane 1 is a control lane for the identification of p25 and p33.

Example 5

Isoelectric Focusing of LT and p33

Figures 9 and 10 (each including an autoradiograph (9A, 10A) and a calibration curve graphing migration distance vs. pH (9B, 10B)) depict isoelectric focusing analysis under denaturing (Fig. 9) and native (Fig. 10) conditions. Two-dimensional gel analysis was carried out as described above on ^{125}I-labeled p25 and p33 that had been immuno- precipitated from II-23.D7 cell extracts. The 2-D gel analysis was performed under denaturing conditions in the presence of urea (Fig. 9A). In contrast, native IEF was performed in 1% NP-40 without urea. ^{125}I-labeled II-23.D7 cell extract was first focused on a tube gel at 4°. After focusing, the tube gel was cut into 1 cm sections, the focused proteins eluted from those sections, immunoprecipitated, and analyzed by SDS-PAGE

(Fig. 10A). Immunoprecipitated material from gel lanes 1-12 correspond to tube gel slices 2-13. pH gradients were generated for both the denatured and native tube gels based on 1 cm gel increments. These are also shown below each autoradiogram as 9B and 10B, respectively.

Example 6

Regulation of LT Expression

Table I set forth below, summarizes the results of a survey using flow cytofluorometric analysis of various cell types for the expression of a surface form of LT.

Table I

Expression of LT and TNF Related Epitopes on the Surfaces of Different Cells

Cell	Treatment	Surface Expression of: LT	TNF
Peripheral Mononuclear cells	Resting	+	-
	OKT3	++	-
Leu-4$^+$ (CD3)	Resting	+	-
	OKT3	++	-
	PMA	-	nd.
	IL-2	++	nd.
Leu-2$^+$ (CD8)	OKT3	++	nd.
Leu-3$^+$ (CD4)	OKT3	++	nd.
Leu-M3	Resting	-	+
	OKT3/LPS/IFN-γ	-	++
Leu-19$^+$ (NK)	IL-2 (LAKs)	++	nd.
Leu-16$^+$ (B's)	Resting	+/-	-
	PWM	-	nd.
CTL-clones	Control	+-	-
	PMA	(?)	nd.
	Allogeneic Stim.	++	nd.
	Anti-T11 2+3	++	-
T cell Hybridoma (II-23.D7)	Control	-	-
	PMA	++	-
	PMA + A23187	++	-
Hut-78	Control	+	-
	PMA	+	-
C8166	Control	-	nd.
	PMA	+	nd.
RPMI-1788	Control	-	-
	PMA	-	-
rLT-producing CHO cell line	Control	-	-
	PMA	-	-
Jurkat		+/-	nd.
HL-60		-	-
U937		-	nd.
Raji		+/-	nd.
K562		+/-	nd.

The most striking observation from these studies was the restriction of surface LT expression to only T cells. Leu-M3, a monocyte marker, and leu-4 (CD3) antibodies were used in two-color flow cytofluorometric analysis to observe each cell population separately. There was an excellent distinction between surface TNF and surface LT in this analysis in that monocytes expressed only surface TNF whereas T cells displayed only surface LT. This result is shown in Figure 11.

In the experiment depicted in Figure 11, PBL were treated for 8 hours with a mixture of LPS (1 μg/ml), Interferon-γ (200 U/ml) and OKT3 (1ng/ml) and then stained for LT (anti-rLT serum from rabbit 5) or TNF (anti-rTNF serum from rabbit 7) followed by FITC anti-rabbit labelling. Cells were counterstained with either phycoerythrin-leu-4, a pan T cell marker, or phycoerythrin leu-M3, a monocyte marker. "T cell panels" were gated for leu-4^+ cells while the "monocyte" panels were gated for leu-$M3^+$ cells. Cells were stained with preimmune (dotted lines) or postimmune sera (solid lines). The monocytic tumor lines HL-60 and U937 did not stain for LT. By using two color flow cytofluorometric analysis, the T4 and T8 subclasses of activated PBL were found to display similar levels of surface-associated LT. In general, it appears that primary T-cells capable of expressing LT are also capable of displaying surface LT form(s).

Examination of three different human donors showed that a surface LT form was present on freshly isolated, resting peripheral T cells. In the case of PBL, OKT3 activation or simply IL-2 treatment of the cells led to increased expression. By using fluorescence channel numbers, we have attempted to quantitate both surface LT and IL-2 receptor (CD25)

expression during OKT3 activation. Maximal surface LT induction by OKT3 appeared to precede the peak expression of IL-2 receptor (TAC expression) in the bulk culture, thus the surface-bound LT form (p33/LT complex) appears to be an early T-cell activation antigen. It was found that both anti-T11 and allogeneic antigen were capable of causing the appearance of LT on the surface of cloned cytotoxic T cells. Likewise, PMA stimulation was necessary to induce the appearance of LT on the surface of the II-23.D7 hybridoma. It appears that T cell activation increases surface LT form(s). Peripheral lymphocytes, in contrast to the II-23.D7 hybridoma, down-regulate surface LT form(s) very quickly following PMA treatment. Likewise, in a two-color analysis of OKT3 activated PBL populations, Dr^+ cells, which should include T cells in advanced stages of activation, lacked surface LT form(s).

Activation of fresh PBL with high levels of IL-2 generated lymphokine-activated killer cells (LAK cells). As shown in Figure 12, two color flow cytofluorometric analysis using anti-rLT and leu-19, a NK/LAK cell marker, showed LAK cell expression of surface LT forms to resemble the T cell hybridoma, II-23.D7. In the experiment depicted in Figure 12, PBL were cultured for 5 days with 20 ng/ml IL-2 and then stained for a two color analysis with phycoerythrin labeled leu-19 and anti-rLT (rabbit 5) as described above. Figure 12 shows surface LT levels on leu-19^+ cells that were stained with preimmune (dotted lines) or postimmune sera (solid lines). Thus, LAK cells appeared to have the highest levels of surface LT forms of any primary cell type.

Example 7

Functional Relevance of Total TNF or LT to T cell Activation

To examine the functional relevance of TNF and LT to the T cell activation process, we included the rabbit anti-rLT and anti-rTNF sera in mixed lymphocyte response (MLR) and OKT3 activation assays. MLR is a standard immunological assay which tests the ability of an individual's T cells to recognize another person's T cells as foreign and respond to their presence by proliferating. Table II, set forth below, presents data from MLR experiments using various responder/stimulator combinations.

TABLE II

Effects of LT and TNF Antibodies
on a 5-Day Mixed Lymphocyte Culture

Cells[b]	Addition	^{3}H-Thy. (S.D.) cpm x 1000	% Change[a]
Responder A	none	4.7 (0.8)	-
Stimulator B*	none	4.8 (0.5)	-
A + B*	none	20.3 (3.6)	0%
A + B*	r-TNF[c]	28.0 (1.4)	+49%
A + B*	r-LT	32.8 (3.2)	+80%
Responder C	none	6.3 (0.8)	-
Stimulator B*	none	4.8 (0.7)	-
C + B*	none	30.0 (5.4)	0%
C + B*	r-TNF	36.9 (6.0)	+28%
C + B*	r-LT	36.1 (5.8)	+24%
Responder A	none	5.3 (0.6)	-
Stimulator D*	none	1.5 (0.5)	-
A + D*	none	22.2 (2.6)	0%
Responder D	none	7.8 (1.0)	-
Stimulator A*	none	1.6 (0.3)	-
D + A*	none	24.3 (7.0)	0%

		Preimmune		Postimmune	
A + B*	Anti-LT-4[d]	26.8 (2.4)	+41%	8.6 (0.2)	-74%
A + B*	Anti-LT-5	27.4 (4.4)	+45%	11.0 (1.2)	-59%
A + B*	Anti-LT-6	23.1 (1.0)	+18%	25.7 (5.7)	+35%
A + B*	Anti-TNF	26.0 (2.2)	+36%	12.6 (2.7)	-49%
A + B*	Anti-TNF mAb*	15.1 (2.2)	-33%	4.7 (0.7)	-99%
C + B*	Anti-LT-4[d]	41.1 (3.5)	+44%	20.9 (5.8)	-36%
C + B*	Anti-LT-5	35.5 (6.9)	+22%	17.8 (2.6)	-49%
C + B*	Anti-LT-6	39.4 (7.9)	+38%	39.1 (5.3)	+36%
C + B*	Anti-TNF	39.8 (4.3)	+39%	24.4 (3.2)	-22%
C + B*	Anti-TNF mAb[e]	37.8 (7.3)	+31%	20.6 (1.8)	-37%
A + D*	Anti-LT-5	28.6 (2.1)	+37%	12.5 (2.4)	-59%
A + D*	Anti-LT-5	32.8 (6.4)	+63%	14.5 (3.6)	-47%
D + A*	Anti-LT-5	28.0 (1.2)	+23%	20.8 (1.4)	-21%
D + A*	Anti-LT-5[f]	28.1 (2.1)	+24%	19.2 (0.7)	-31%

See notes below.

[a]Percent change refers to the increase or decrease in ^{3}H-thymidine incorporation after correction for a background of responder cells alone.

[b]Stimulator cells were irradiated with 3000 rads and are denoted with a "*". Low level proliferation was still evident in stimulator population. The ratio of responder to stimulator was 1/1.5.

[c]rTNF and rLT were added to a level of 10 ng/ml.

[d]Antisera were heat inactivated for 1 hour at 56°C, filtered and used at a final dilution of 1:250.

[e]The monoclonal anti-TNF was a purified mouse IgG_{2a} antibody used at 2 μg/ml and the control in this case was pure mouse UPC 10 (IgG_{2a}).

[f]In these cases, the immunoglobulin fraction was purified from the serum and used at a final concentration of 50 μg/ml.

As shown in Table II, the neutralizing anti-rLT sera (rabbits 4 and 5) inhibited the proliferative response as assessed at five days whereas preimmune sera or the non-neutralizing anti-rLT (rabbit 6) sera had mild stimulatory effects. As previously reported [M. Shalaby et al., "The Involvement Of Human Tumor Necrosis Factors-α And -β In The Mixed Lymphocyte Reaction", J. Immun., 141, 499 (1988)], polyclonal and a monoclonal anti-TNF preparations were also inhibitory. These assays were carried out under excess stimulator cell conditions and hence the inhibition may not be optimized. The serum levels employed in Table II are rather high, but in other experiments (data not shown), antibody dilutions up to 1:1000 were still inhibitory. PHA or OKT3 stimulated T cell proliferation was also inhibited to a lesser extent (data not shown). These data indicated that LT or LT-related epitopes on T cell surfaces may be involved in T cell activation.

A previous study using the MLR assay and neutralizing monoclonal antibodies implicated TNF but not LT in T cell activation and subsequent proliferation in this system. [M. Shalaby et al., supra]. In that study, monoclonal anti-rLT antibodies had no effect on the MLR assay. Our studies indicate that neutralizing polyclonal anti-CHO-cell-derived-rLT sera were able to partially inhibit the MLR, suggesting a role for some form of LT in this system. The reasons for this discrepancy are not clear, although there may be some differences in the nature of these antibody preparations. The monoclonal antibodies were generated against glutaraldehyde-cross linked natural LT (RPMI 1788 secreted), whereas polyclonal anti-rLT sera were prepared using native r-LT (recombinant CHO-cell derived) with Freund's adjuvant injected directly into the lymph nodes. The depot effect in the success of the latter system was probably important considering the difficulties reported in immunizing mice [T. Bringman et al., "Monoclonal Antibodies to Human Tumor Necrosis Factors Alpha and Beta: Application For Affinity Purification, Immunoassays, And As Structural Probes", Hybridoma, 6, 489 (1987)]. These data suggest that the blocking effect of our polyclonal anti-rLT sera on the MLR is a result of recognition of the surface LT form(s) by the sera rather than recognition of the conventional LT form.

Example 8

Purification of LT and the Associated Protein p33

We grew II-23.D7 cells in RPMI medium with 10% fetal bovine serum and we harvested the cells from 50 1 RPMI medium and resuspended them in medium at a concentration of 4×10^6 cells/ml and we added 50 ng/ml

phorbol myristoyl acetate (PMA). After activation for 6 hours we harvested the cells by centrifugation and washed them with Dulbecco's phosphate buffered saline. We suspended the final cell pellet of 4 x 10^{10} cells in 200 ml of cold lysis buffer (50 mM HEPES buffer, pH 7.0; 0.1 M NaCl, 10 mM EDTA, 5 mM benzamidine, 10 µg/ml each of soybean trypsin inhibitor, aprotinin, chymostatin, leupeptin, antipain, 1 µg/ml pepstatin and 1 mM phenylmethyl sulfonyl fluoride) and passed the pellet once through a nitrogen cavitator. We centrifuged the lysed the cells at 40,000 rpm for 60 minutes in a 50.2 Ti rotor and discarded the supernatent. We extracted the pellet overnight in 120 ml of lysis buffer with 1% w/v Nonidet P40 detergent and then centrifuged it as above.

We added the supernatent containing the detergent solubilized membrane proteins to 2 ml of affinity resin composed of monoclonal anti-lymphotoxin (anti-tumor necrosis factor-ß from Boehringer Mannheim) coupled to Affi-gel 10 (BioRad) and rocked the suspension overnight. We collected the resin into a small column and washed it with 50 mM HEPES, pH 7.0 with 1% Nonidet P40, and then with the same buffer with 1% w/v MEGA-8 (Boehringer Mannheim). We eluted the bound proteins with 1% MEGA-8 in 50 mM glycine buffer pH 2.5 and the fractions immediately neutralized with Tris base. We determined the presence of p33 and LT in the fractions by SDS-PAGE analysis and silver staining. We pooled factions containing these proteins and added SDS to a final concentration of 0.1% w/v and we dialyzed the pool against 0.1x Laemmli sample buffer (multiple changes to remove the MEGA-8 detergent). We lyophilized the dialyzed solution to dryness and resuspended it in 1/10th the original volume of water. We ran the sample on an SDS-PAGE gel, blotted onto a

ProBlot membrane (Applied Biosystems) and stained with coomassie blue dye. We excised the p33 and 25 kD LT bands and loaded them into a protein sequencer. We obtained N-terminal sequence by Edman degradation with a model 470A Applied Biosystems sequencer coupled to a 120A PTH amino acid analyzer. We found the sequence of the membrane-associated lymphotoxin band to exactly match that described for secreted lymphotoxin, i.e., (SEQ ID NO:1) Leu Pro Gly Val Gly Leu Thr Pro Ser. The N-terminal sequence of the protein of the p33 band was (SEQ ID NO:2) Gly Leu Glu Gly Arg Gly Gln Arg Leu Gln or (SEQ ID NO:3) Gly Leu Glu Gly Arg Leu Gln Arg Leu Gln. The sequence at the 6th cycle appeared to be a mixture of both Gly and Leu, indicating possible polymorphism. The p33 protein may have one of these sequences or both.

This scheme allows one to purify p33 to a band on a blot. It should be possible for anyone skilled in the art to separate the proteins eluted from the affinity resin by ion exchange chromatography. For example, the complex can be dissociated with urea and the LT and p33 proteins can be separated by, e.g., MONO Q FPLC (Pharmacia) anion exchange chromatography in Tris-Cl buffer pH 8.0 with 1% nonionic detergent (e.g., MEGA-8, Boehringer-Mannheim) and urea, using a salt gradient elution. This chromatographic technique separates on the basis of differing charges on the proteins. The two proteins are separable in an isoelectric focusing experiment (see, supra) on the basis of charge differences, wherein urea is used to dissociate the p33/LT complex. Such a combination of affinity chromatography, dissociation in urea/nonionic detergent and ion exchange chromatography allows purification of soluble p33 or the p33/LT complex.

Example 9

Cloning Strategies

A: The protein sequence allows one to predict possible nucleic acid sequences that would encode this protein. To clone p33, we are taking pools of a 17 mer oligonucleotide sequence that encode a 6 amino acid segment. In principle any segment can be employed. Radiolabeled oligonucleotide pools will be used to probe northern blots of RNA from PMA-induced II-23.D7 and Hut-78 (no PMA) both of which must express p33 as well as RNA from several non-hematopoietic lines, e.g. HT1080, ME-180, MCF-7 and RL-95. Since LT is only observed on T cells even though other cells can secrete LT (but not direct it to the surface), we are postulating that p33 is only expressed in T cells. We will look for mRNA's that are expressed preferentially in T cells that are larger than about 1.0 kb (to code for a 30 kD protein). When we find the right probe pool we will use that probe to screen a PMA induced II-23.D7 cDNA library. The correct clone will target LT to the surface in a CHO cell line that is stably transfected with rLT and constitutively secretes LT [Browning and Ribolini, J. Immunol., supra].

B: Both LT and p33 are required to obtain the p33/LT complex, and thus normal expression cloning strategies will not work since the likelihood of both clones being transfected into the same cell is very low. Transfection of mammalian cells such as COS cells with a cDNA library from II-23.D7 and also LT cDNA in expression vectors should direct LT to the surface in those cells transfected with both LT and p33 DNA. Transfection of COS cells with LT alone will not lead to appearance of a surface LT form. The cells expressing both LT and p33 could then be panned on anti-LT antibodies. [A. Aruffo and B. Seed, "Molecular

Cloning of a CD28 cDNA by a High Efficiency COS Cell Expression System", Proc. Natl. Acad. Sci. USA, 84, 8573-77 (1987).] The PMA-induced II-23.D7 cell line pans very well in this situation. Hence, we expect screening a PMA-induced II-23.D7 library in the pCDM8 vector system on the LT transfected CHO cell will lead to the clone for p33.

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(B) TELEFAX: 212-715-0674
(C) TELEX: 14-8367

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Leu Pro Gly Val Gly Leu Thr Pro Ser
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly Leu Glu Gly Arg Gly Gln Arg Leu Gln
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gly Leu Glu Gly Arg Leu Gln Arg Leu Gln
1 5 10

Claims

We claim:

1. A polypeptide that is not cell associated comprising an amino acid sequence selected from the group consisting of (SEQ ID NO:2) Gly Leu Glu Gly Arg Gly Gln Arg Leu Gln and (SEQ ID NO:3) Gly Leu Glu Gly Arg Leu Gln Arg Leu Gln in its amino terminal portion.

2. The polypeptide according to claim 1, further having a molecular weight of between 31 to 35 kD.

3. A DNA consisting essentially of a DNA sequence encoding on expression a polypeptide comprising an amino acid sequence selected from the group consisting of (SEQ ID NO:2) Gly Leu Glu Gly Arg Gly Gln Arg Leu Gln and (SEQ ID NO:3) Gly Leu Glu Gly Arg Leu Gln Arg Leu Gln in its amino terminal portion.

4. A recombinant DNA molecule comprising a DNA sequence encoding on expression a polypeptide comprising an amino acid sequence selected from the group consisting of (SEQ ID NO:2) Gly Leu Glu Gly Arg Gly Gln Arg Leu Gln and (SEQ ID NO:3) Gly Leu Glu Gly Arg Leu Gln Arg Leu Gln in its amino terminal portion.

5. A host selected from the group of unicellular hosts or animal and human cells in culture, transfected with a DNA sequence encoding on expression a polypeptide comprising an amino acid sequence selected from the group consisting of (SEQ ID NO:2) Gly Leu Glu Gly Arg Gly Gln Arg Leu Gln and (SEQ ID NO:3) Gly Leu Glu Gly Arg Leu Gln Arg Leu Gln in its amino terminal portion.

6. The host according to claim 5 selected from the group of tumor infiltrating lymphocytes, lymphokine activated killer cells and killer cells.

7. A method for producing a polypeptide comprising an amino acid sequence selected from the group consisting of (SEQ ID NO:2) Gly Leu Glu Gly Arg Gly Gln Arg Leu Gln and (SEQ ID NO:3) Gly Leu Glu Gly Arg Leu Gln Arg Leu Gln in its amino terminal portion, the method comprising the steps of culturing a transformed host according to claim 5 and collecting the polypeptide.

8. A polypeptide complex that it not cell associated comprising a polypeptide that comprises an amino acid sequence selected from the group consisting of (SEQ ID NO:2) Gly Leu Glu Gly Arg Gly Gln Arg Leu Gln and (SEQ ID NO:3) Gly Leu Glu Gly Arg Leu Gln Arg Leu Gln in its amino terminal portion and a lymphotoxin.

9. The complex according to claim 8, wherein the non-lymphotoxin polypeptide has a molecular weight of 31 to 35 kD.

10. A polypeptide complex that is not cell associated comprising a polypeptide produced according to the method of claim 7 and a lymphotoxin.

11. The complex according to any one of claims 8 to 10, wherein the lymphotoxin is selected from the group of native human or animal lymphotoxin, recombinant lymphotoxin, soluble lymphotoxin, secreted lymphotoxin or lymphotoxin-active fragments of any of the above.

12. A method for increasing the amount of lymphotoxin epitopes on the surface of a cell that produces and secretes those epitopes comprising the steps of transfecting the cell with a DNA sequence according to claim 3 and expressing that DNA in the cell.

13. A method for enhancing the targeting tumorcidal activity of tumor infiltrating lymphocytes

comprising the steps of transfecting the lymphocytes with a DNA sequence according to claim 3 and introducing the transformed lymphocytes to a patient.

14. The method according to claim 13, wherein the transformed lymphocytes are incubated with a lymphokine before or after transfection with the DNA sequence according to claim 3.

15. The method according to claim 14, wherein the lymphokine is IL-2.

16. The method according to any one of claims 13 to 15, wherein the lymphocytes are initially isolated from and then introduced back to the same patient.

17. A composition for preventing or lessening the spread, severity or immunocompromising effects of HIV infection comprising an effective amount of a polypeptide selected from the group consisting of a polypeptide according to claim 1 or 2, a polypeptide complex according to any one of claims 8 to 11, and antibodies to any of the above, and a pharmaceutically acceptable carrier.

18. A method for preventing, treating or lessening the spread, severity or effects of HIV infection comprising administering an effective amount of a polypeptide selected from the group consisting of a polypeptide according to claim 1 or 2, a polypeptide complex according to any one of claims 8 to 11, and antibodies to any of the above, and a pharmaceutically acceptable carrier.

19. A composition for preventing, treating or lessening the spread, severity or effects of neoplasia comprising an effective amount of a polypeptide selected from the group consisting of a polypeptide according to claim 1 or 2, a polypeptide complex according to any one of claims 8 to 11, and

antibodies to any of the above, and a pharmaceutically acceptable carrier.

20. A method for preventing, treating or lessening the spread, severity or effects of neoplasia comprising administering an effective amount of a polypeptide selected from the group consisting of a polypeptide according to claim 1 or 2, a polypeptide complex according to any one of claims 8 to 11, and antibodies to any of the above, and a pharmaceutically acceptable carrier.

21. A composition for preventing, treating or lessening the spread, severity or effects of inflammation or inflammatory diseases comprising an effective amount of a polypeptide selected from the group consisting of a polypeptide according to claim 1 or 2, a polypeptide complex according to any one of claims 8 to 11, and antibodies to any of the above, and a pharmaceutically acceptable carrier.

22. A method for preventing, treating or lessening the spread, severity or effects of inflammation or inflammatory diseases comprising administering an effective amount of a polypeptide selected from the group consisting of a polypeptide according to claim 1 or 2, a polypeptide complex according to any one of claims 8 to 11, and antibodies to any of the above, and a pharmaceutically acceptable carrier.

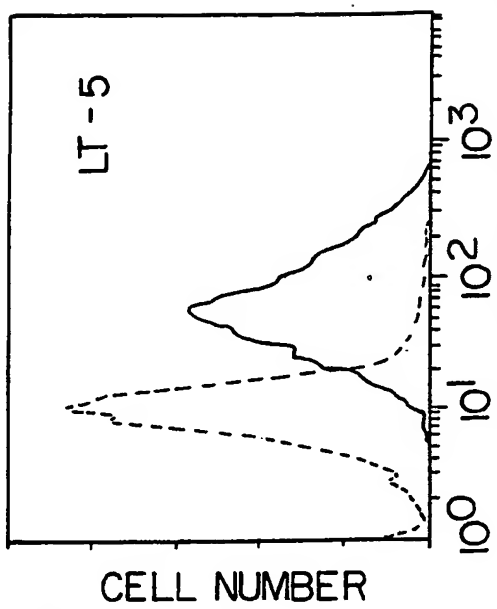


FIG. 1B

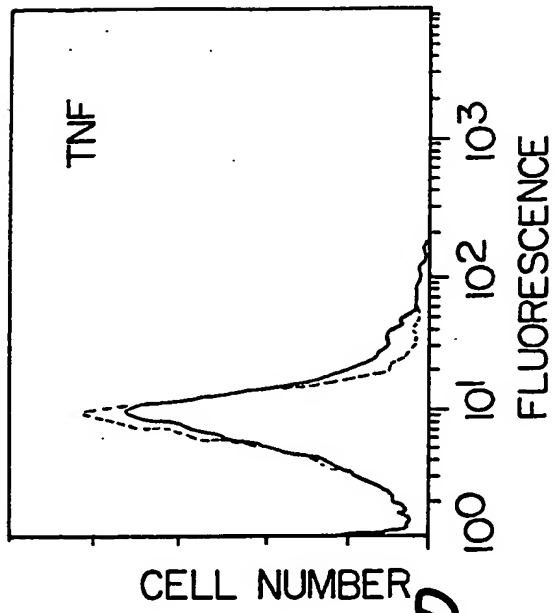


FIG. 1D

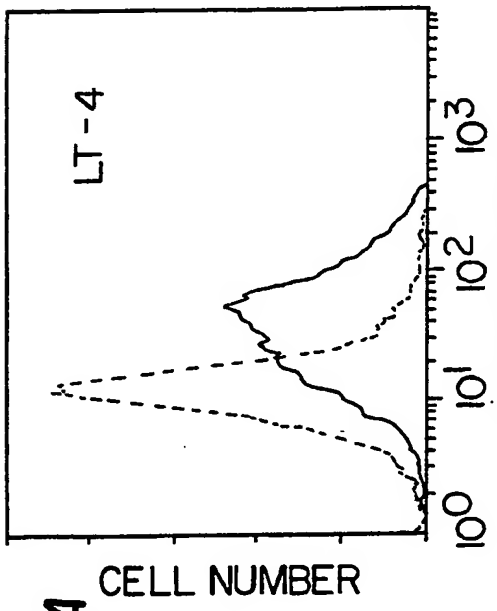


FIG. 1A

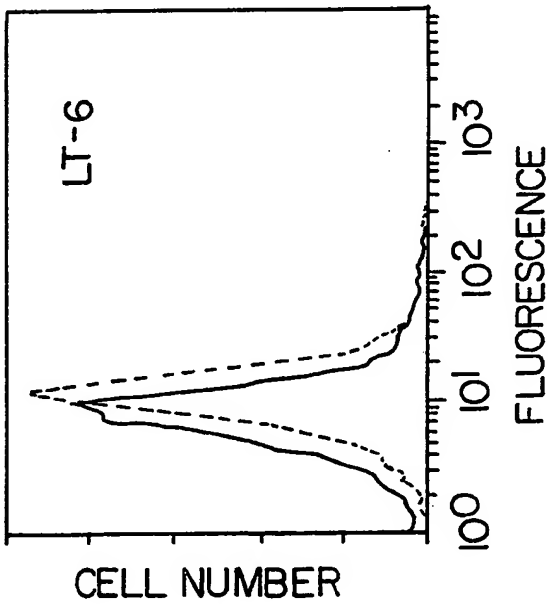


FIG. 1C

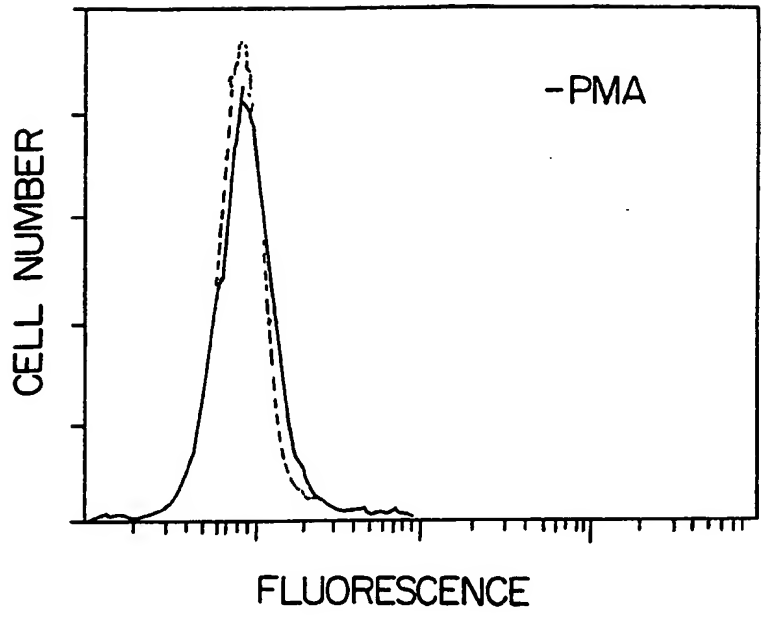

-PMA
CELL NUMBER
FLUORESCENCE

FIG. 2A

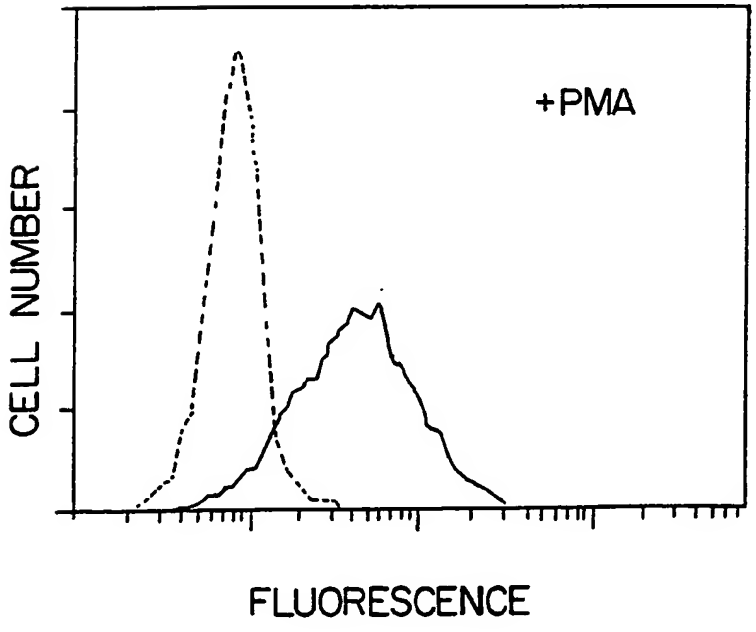

+PMA
CELL NUMBER
FLUORESCENCE

FIG. 2B

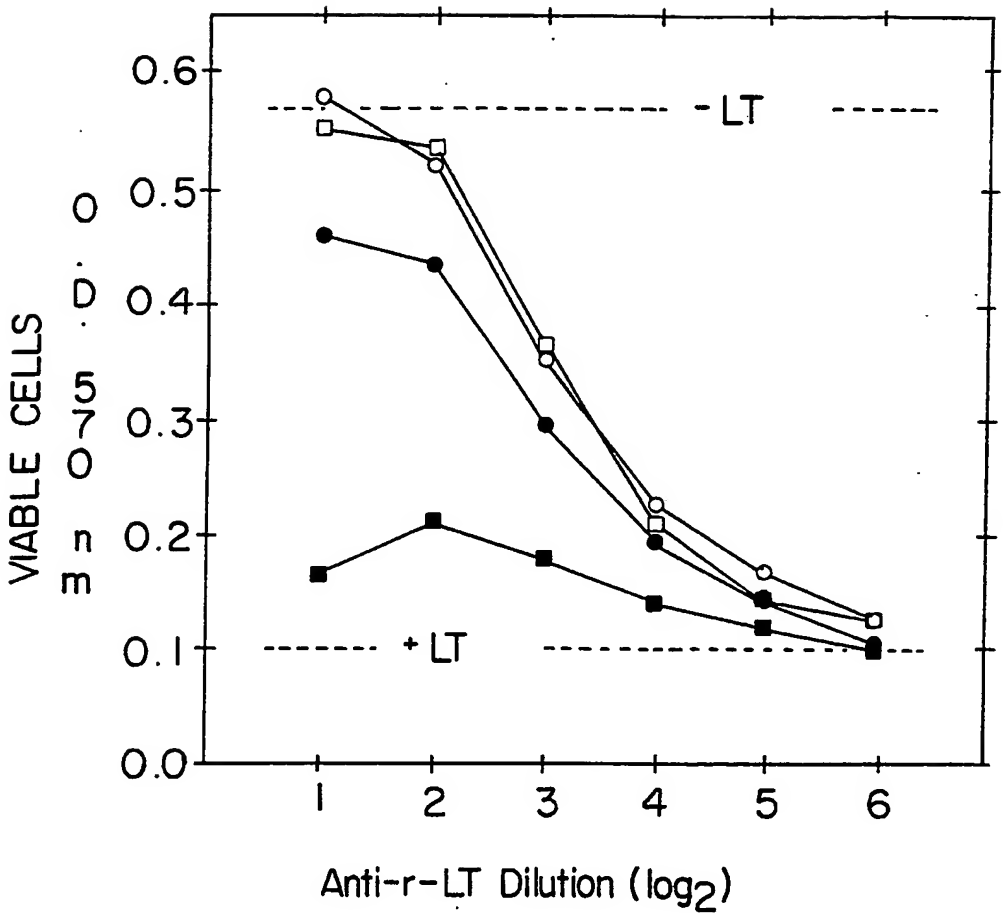

VIABLE CELLS
m n O.D. 595
0.6
0.5
0.4
0.3
0.2
0.1
0.0
- LT
+ LT
1
2
3
4
5
6
Anti-r-LT Dilution (log2)

FIG. 3

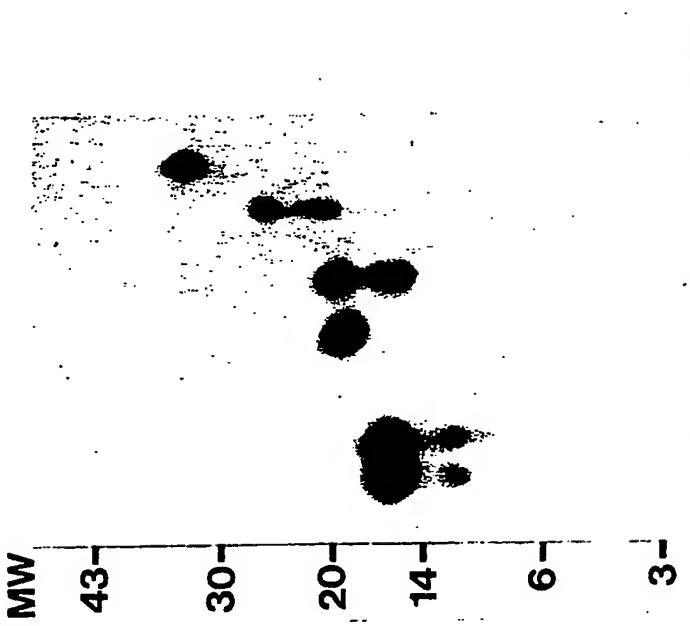

MW
43
30
20
14
6
3
- + - + + + CNBr
rTNF rLT sLT p33

FIG. 4B

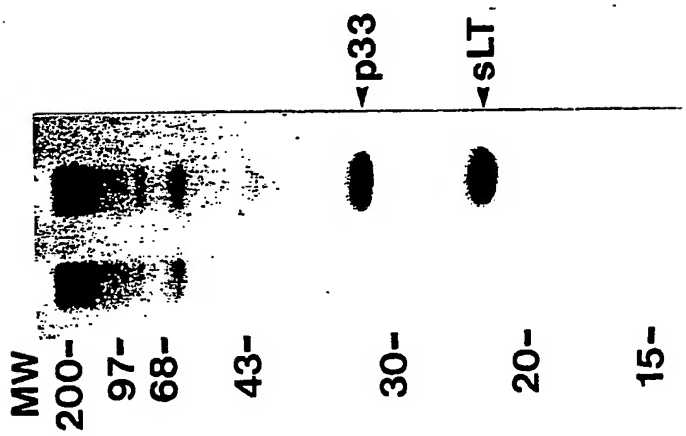
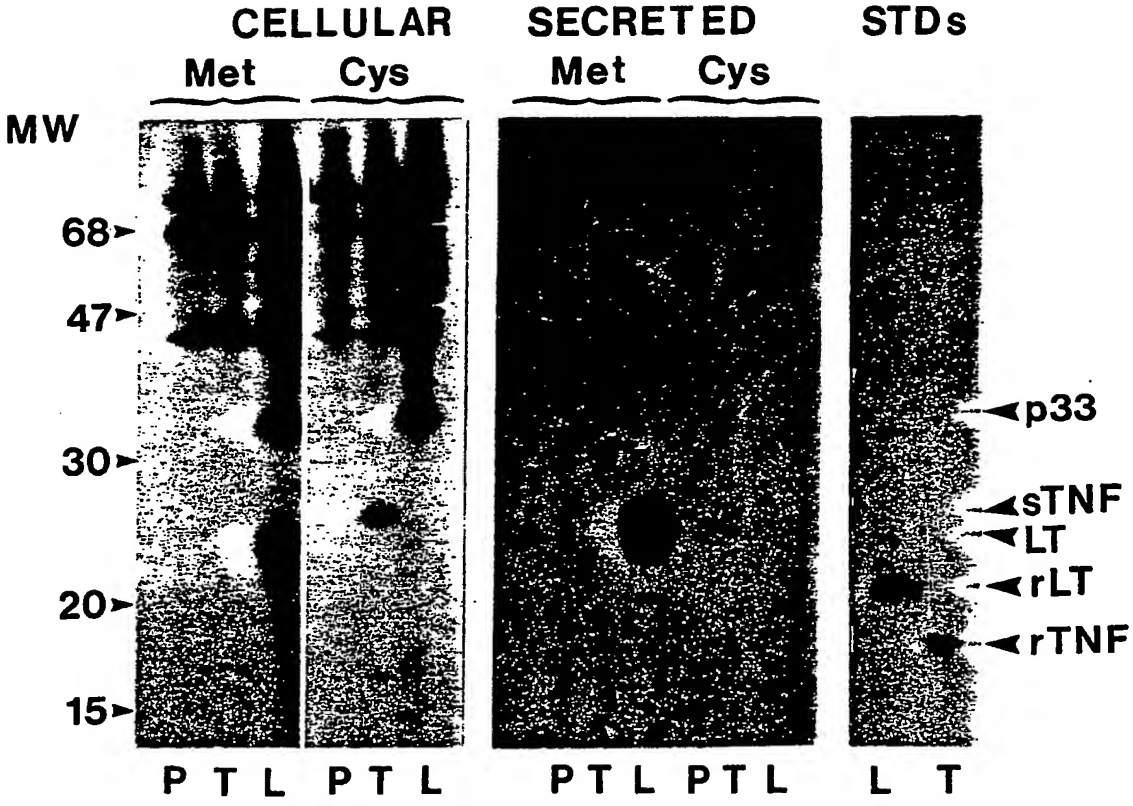

p33
sLT
MW
200
97
68
43
30
20
15
PRE POST

FIG. 4A

FIG. 5


CELLULAR
SECRETED
STDs
Met
Cys
Met
Cys
MW
68
47
30
20
15
p33
sTNF
LT
rLT
rTNF
P T L P T L
P T L P T L
L T

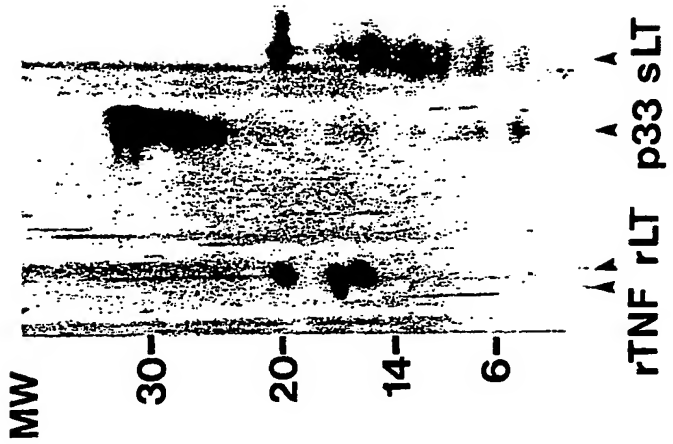


FIG. 6B

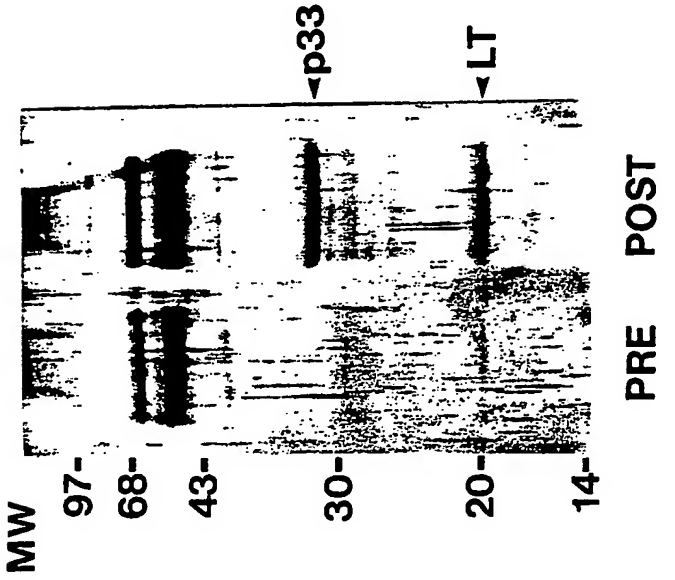


FIG. 6A

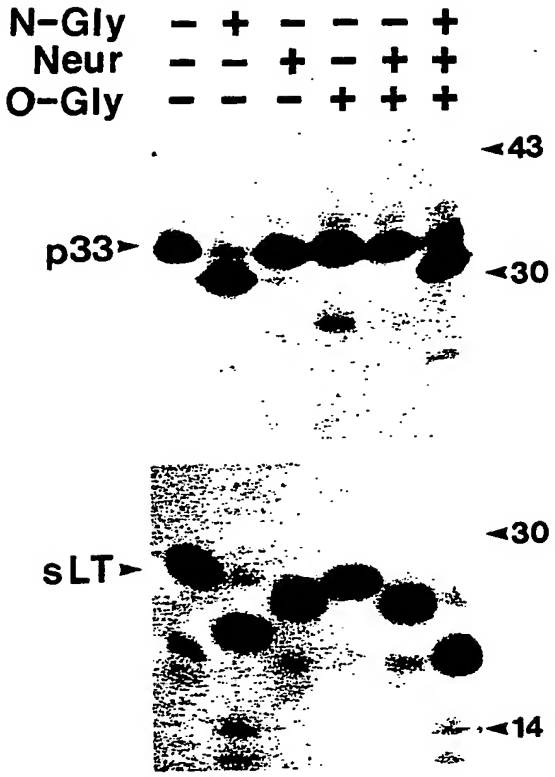


FIG. 7

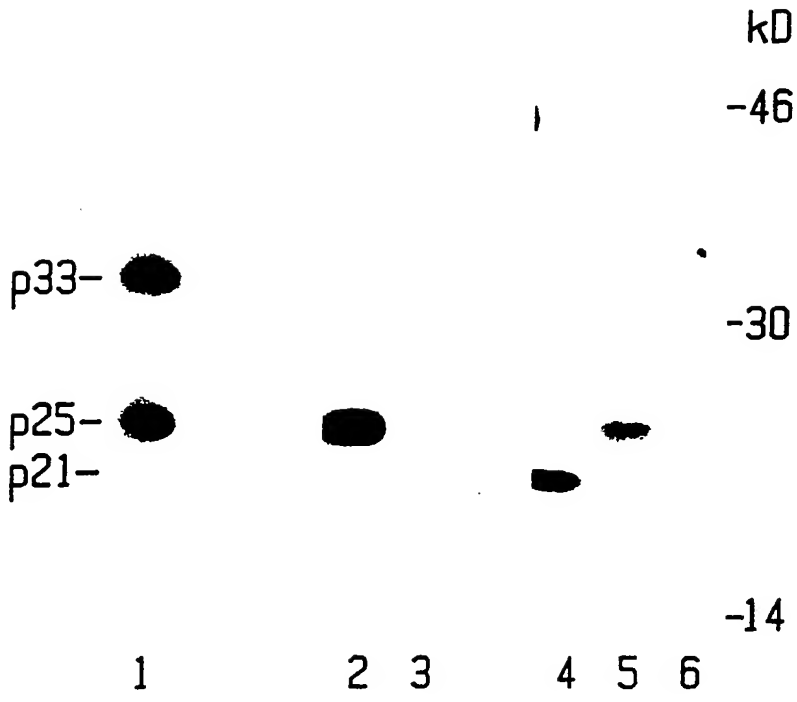


FIG. 8

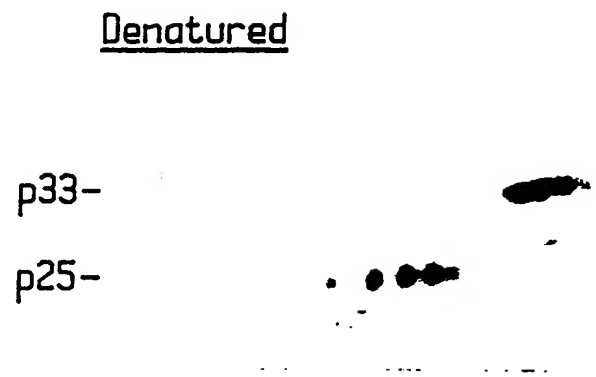


FIG. 9A

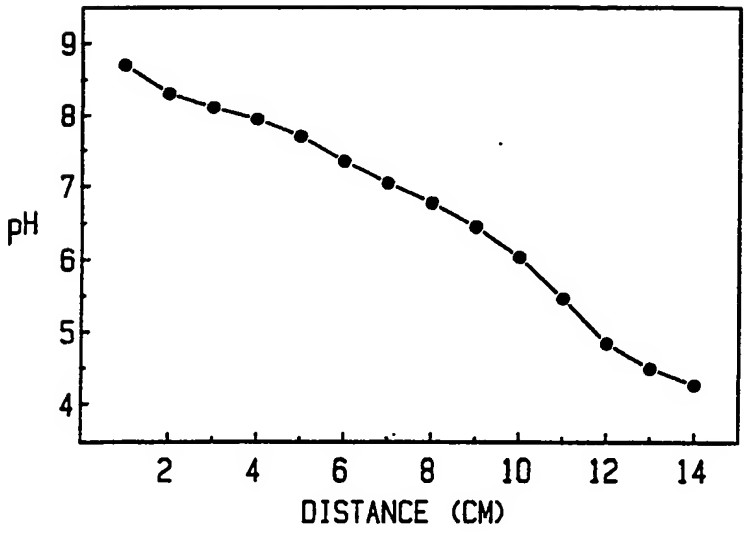


FIG. 9B

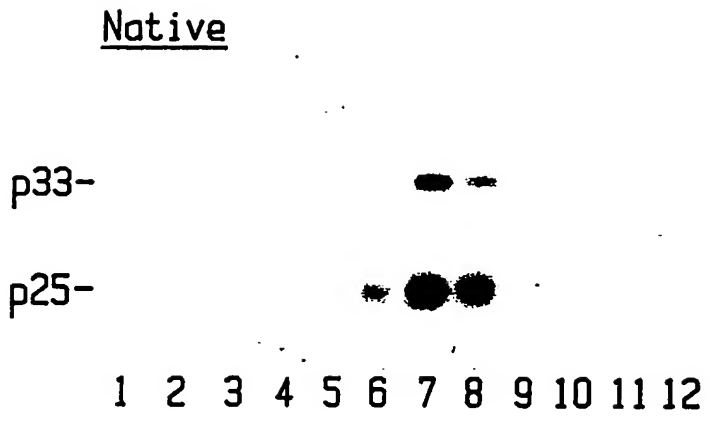


FIG. 10A

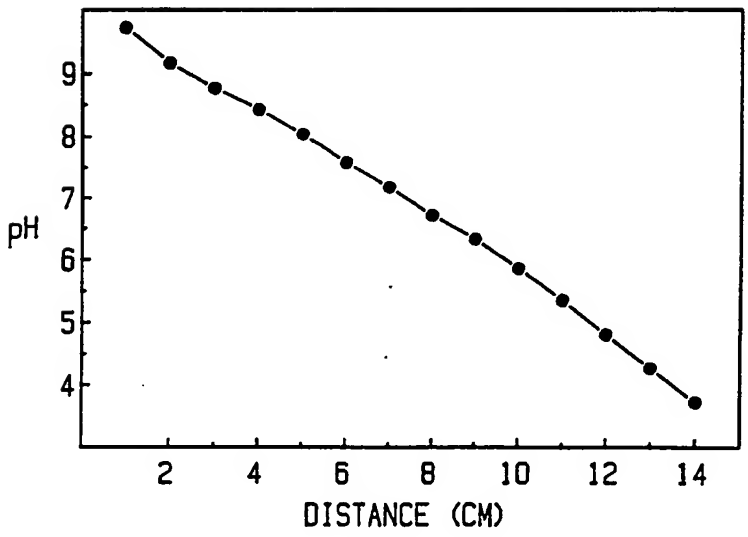


FIG. 10B

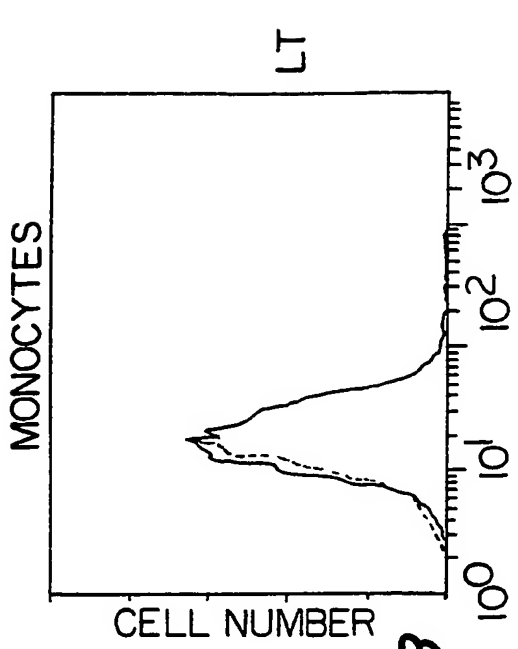


FIG. 11B

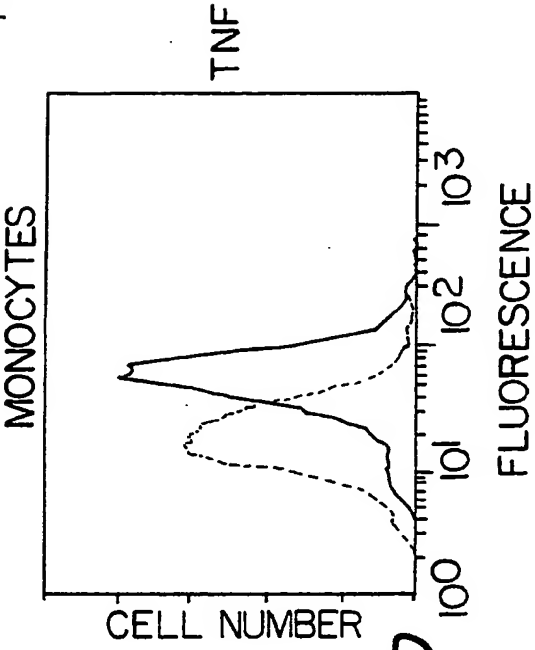


FIG. 11D

FIG. 11A

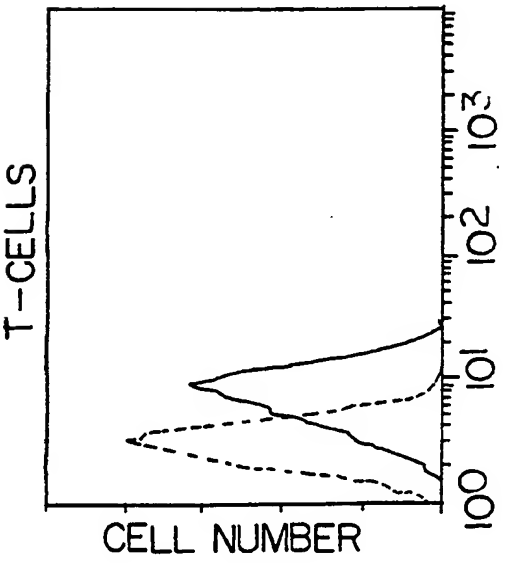
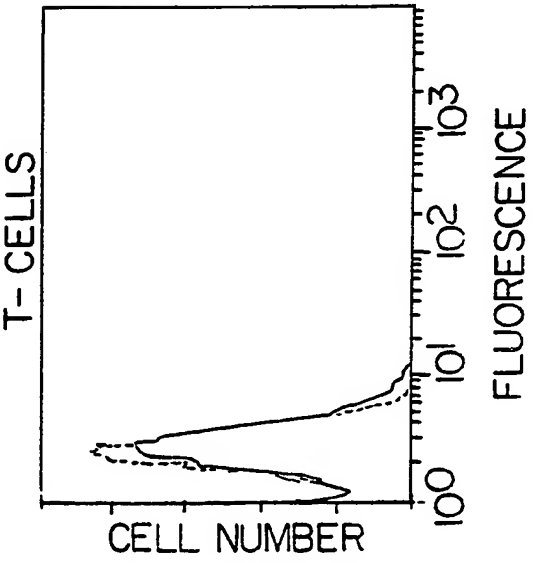


FIG. 11C



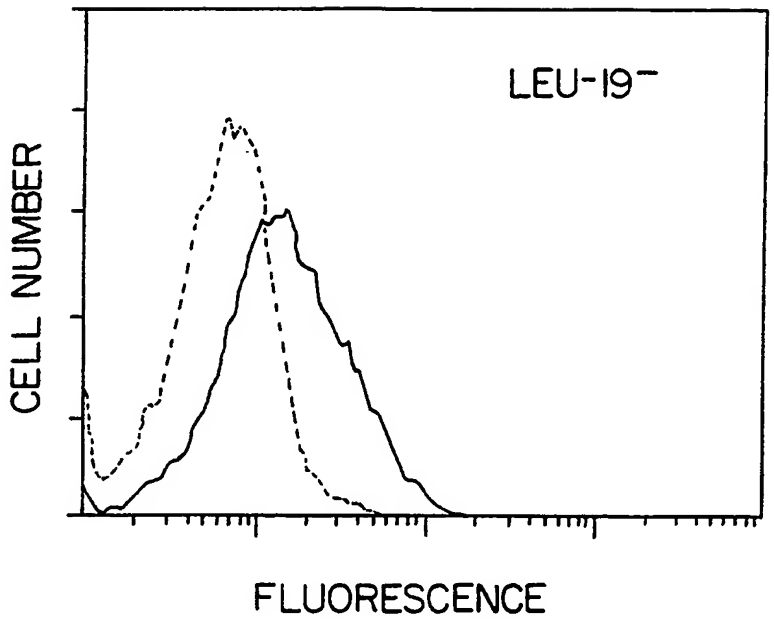


FIG. 12A

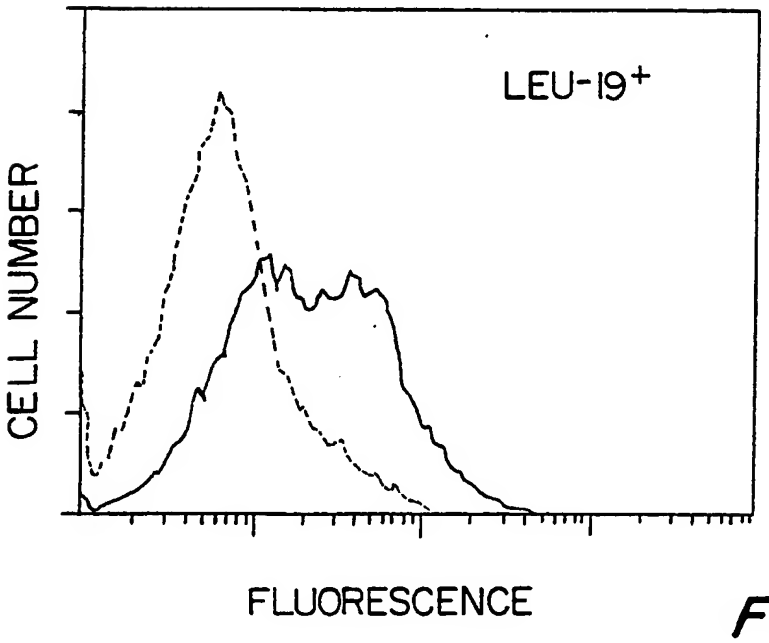


FIG. 12B

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/04588

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 15/06, C 12 N 15/12, A 61 K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 07 K; C 12 N; A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
E	Dialog Information Services, File 154, Medline 85-89, Dialog accession no. 07813432, Browning JL et al: "Lymphotoxin and an associated 33-kDa glycoprotein are expressed on the surface of an activated human T cell hybridoma", & J Immunol (UNITED STATES) Aug 15 1991, 147 (4) p1230-7 --	1-17,19, 21
A	Dialog Information Services, File 154, Medline 85-91 Dialog accession no. 07296587, Andrews JS et al: "Characterization of the receptor for tumor neorosis factor (TNF) and lymphotoxin (LT) on human lymphocytes. TNF and LT differ in their receptor binding properties and the induction of MHC class I proteins on a human CD4+ T cell hybridoma published erratum appears in J Immunol 1990 Jul 15;144(12):4906A, & J Immunol Apr 1 1990, 144 (7) p 2582-91 --	1-17,19-21

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11th October 1991	20. 11. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Maria TORIBIO

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Dialog Information Services, File 154, Medline 85-91 Dialog accession no. 07075928, Gullberg U et al: "Characterization of the receptor for lymphotoxin; a spontaneous internalization without recycling and ligand-induced downregulation in HL-60 cells", & Eur J Cell Biol Aug 1989, 49 (2) p334-40 --	1-7,19, 21
A	Dialog Information Service, File 154, Medline 85-91, Dialog accession no. 06989792, Hirano K et al: "Characterization of specific high-affinity receptor for human lymphotoxin", & J Biochem (Tokyo) Jan 1989 105 (1) p120-6 --	1-12,19, 21
A	US, A, 4770995 (BERISH Y. RUBIN ET AL.) 13 September 1988, see the whole document --	1-17,19, 21

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 18, 20, 22 because they relate to subject matter not required to be searched by this Authority, namely:

Methods for treatment of the human or animal body c.f. PCT rule 39. iv.

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additonal fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional seach fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 91/04588

SA 49522

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 30/08/91
The European Patent office is in no way liable for theseparticulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4770995	13/09/88	NONE	

For more details about this annex : see Official Journal of the European patent Office, No. 12/82

EPO FORM P0479